Cloning And Expression Of A Novel Neural Gene

by
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Declaration

I hereby declare that the composition and experiments of this thesis are my own, unless stated otherwise. No part of this work has been, or is being, submitted for any other degree or qualification.

Signed Date

18/11/97

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LIST OF ABREVIATIONS

A	Amps
Arg	Arginine
Asp	Aspartic acid
ATP	Adenosine triphosphate
bFGF	Basic fibroblast growth factor
bp	Base pair
^{o}C	degrees centigrade
cDNA	Coding DNA
Ci	Curies
CNPase	2´,3´-cyclic nucleotide 3´-phosphohydrolase
CNS	Central nervous system
CNTF	Ciliary neurotrophic factor
CTP	cytidine triphosphate
cpm	Counts per minute
DEPC	Diethylpyrocarbonate
dH_2O	Distilled water
DMEM	Dulbecco's Modified Eagle's Medium
DNA	Deoxyribonucleic acid
dNTP	Deoxy nucleotide triphosphate
DTT	Dithiothreitol
E	Embryonic day
EDTA	Ethylenediaminetetraacetic acid
FCS	Foetal calf serum
GC	Galactocerebroside
GFAP	Glial fibrilliary acidic protein
GGF	Glial growth factor
Gln	Glutamine
Glu	Glutamic acid
Gly	Glycine
GTP	Guanosine triphosphate
h	Hours
HBSS	Hank's balanced salt solution
IGF	Insulin like growth factor
ISH	in situ hybridisation
kb	Kilobase
kD	Kilodalton
M	Molar
MAG	Myelin associated glycoprotein
mBar	MilliBar
MBP	Myelin basic protein
min	Minute
mg	milligram
µg	microgram
ml	millilitre
µl	microlitre
MOBP	Myelin-associated/oligodendrocytic basic protein
MOG	Myelin/oligodendrocyte glycoprotein
MOSP	Myelin/oligodendrocyte specific protein
mRNA	Messenger RNA
MW	Molecular weight
ng	nanogram

nm	nanometer
NT3	Neurotrophin 3
O-2A	Oligodendrocyte-type 2 astrocyte
OD	Optical density
OMgp	Oligodendrocyte myelin glycoprotein
P	Postnatal day
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PDGF	Platelet derived growth factor
pfu	Plaque forming units
pI	Isoelectric point of a protein
PLP	Proteolipid protein
pMol	Picomole
PNS	Peripheral nervous system
RNA	Ribonucleic acid
rpm	Revolutions per minute
RT	Room temperature
Ser	Serine
SDS	Sodium dodecyl sulfate
SSC	Salt, sodium citrate
TEMED	N,N,N',N'-Tetramethylethylenediamine
TGF	Transforming growth factor
Thr	Threonine
TRITC	Tetramethyl Rhodamine Isothiocyanate
TTP	Thymidine triphosphate
U	Unit
UTP	Uridine triphosphate
UV	Ultraviolet
V	Volt
v/v	Volume by volume
w/v	Weight by volume

ABSTRACT

Subtractive hybridisation is a powerful method for isolating and identifying rare genes with an expression pattern restricted to specific cell types. Five novel cDNA clones were isolated and identified from a differentiated glial subtractive library. They were shown to be specific to the nervous system by Northern blotting analysis. In situ hybridisation (ISH) studies were carried out on cultured glial cells to determine if any of the clones were expressed by specific glial cell types. Oligodendrocytes were identified by the monoclonal antibody O4 and astrocytes were identified with an anti-GFAP monoclonal antibody.

Clone OL0755, one of the novel brain specific clones identified, was chosen for further investigation. Two cDNA clones, believed to be full length, were obtained by hybridisation screening of a rat cDNA library. Clone OL0755-A is 2.7 kb and encodes a protein of approximately 60 kD. Clone OL0755-B is 2 kb in size and encodes a protein of approximately 50 kD. Sequence analysis showed these clones to be two alternatively spliced forms of a common gene. An antibody (Ab755) raised against a 23 amino acid N-terminus peptide common to both clones (EASFVQTTMALGLPSKKASSRNV) identifies two proteins with the predicted sizes, 50 kD and 60 kD. Both the mRNAs and the proteins are developmentally regulated. Both mRNAs increase in abundance in the postnatal rat brain peaking at postnatal day 21 (P21). Expression of the smaller, more abundant protein also increases during development peaking at P21 while the larger protein is less abundant and downregulates slightly with age. Neither protein is phosphorylated or N-glycosylated. ISH studies on P21 rat brain sagittal sections with clone OL0755 (common to both OL0755-A and OL0755-B) showed a very strong neuronal pattern of expression but also a weaker expression in glial cells which was confirmed by ISH on rat optic nerves and which was consistent with the ISH studies on cultured glial cells. The appearance of these proteins in both glial cells and neurons during the active phase of myelination suggests a possible role in this process either as a structural component or as part of a signalling mechanism between the cell types.

CHAPTER 1. INTRODUCTION

The nervous system of higher vertebrates is a network of electrically conducting cells which relays information in a very fast and efficient manner between the brain and all parts of the body. It divides into two parts: the central nervous system (CNS) which is composed of the brain and spinal cord and the peripheral nervous system (PNS) which encompasses the rest of the body. The electrically conducting cells which relay the information are the neurons. These cells have long processes, called axons, extending from their cell bodies which transmit the information as electrical impulses. Interconnected neurons form networks of routes for the information to travel around the body to and from the brain. A group of cells in close proximity to the neuronal networks, called the neuroglia, play a supporting role for the neurons.

1.1 Myelination

Many axons in the CNS and PNS of vertebrates are ensheathed by a multilayered myelin membrane which acts as an electrical insulator. In unmyelinated nerve fibres a nerve impulse is propagated as an action potential which travels along the axon as a wave of membrane depolarisation. A myelin sheath insulates the axon from the extracellular fluid and prevents the transport of ions across the axonal membrane. This creates a region of high electrical resistance. An action potential can therefore only be generated at nodes of Ranvier. These are small regions of unmyelinated axon in contact with the extracellular fluid which are present at intervals along the axon between the myelin sheaths. The action potential jumps as an electrical current from one node to the next. This very fast form of impulse propagation is therefore known as saltatory conduction (from the Latin *saltare*, to jump). Equivalent conduction velocities in unsheathed axons are only possible if axonal diameter increases since the rate of impulse transmission in unmyelinated fibres is proportional to the square of the fibre diameter. Therefore to increase the rate of conduction four-fold, a fibre diameter must increase $4^2 = 16$ times. Only an enormous unmyelinated invertebrate axon (e.g. squid giant axons) with a diameter of 1mm can approach a conduction velocity of 100 meters per second achieved by a myelinated human axon with diameter of 20 μm. Also, since ion exchange only occurs at nodes of Ranvier and not all over the membrane, myelinated fibres use less than 1% of the energy required by unmyelinated fibres to conduct an impulse. Therefore since myelination permits several hundred axons in the space of one squid giant axon each achieving high conduction velocities at low energy cost, this insulation material is extremely important to the nervous system by minimising the space it requires whilst maximising impulse velocities.

In the CNS the cells responsible for producing this insulation material are the oligodendrocytes. Oligodendrocytes are one of the main types of macroglia, a subgroup of the neuroglia, and they have sole responsibility for producing and maintaining the myelin sheaths. (The oligodendrocytes counterpart in the PNS is the Schwann cell). This relationship between oligodendrocytes and neurons was recognised as early as 1900 by Holmgren and by Cajal in 1919 (Cajal, 1919; Holmgren, 1900) who suggested that the neuroglia might have an insulator function. This idea was later substantiated by the student of Ramon y Cajal, del Rio Hortega (del Rio Hortega, 1928). The sheath is produced from the extended processes of oligodendrocytes which flatten and widen as myelin membrane forms and envelops sections of nearby axons. These sections of myelinated axon are the internodes, interspersed by the nodes of Ranvier which allow saltatory conduction of impulses along the nerve. Some single oligodendrocytes have been shown to extend as many as 50 processes each myelinating individual internodes on multiple axons at the one time (Pfeiffer et al., 1993). Compaction of the myelin occurs as the membrane spirals around the axon. The cytoplasm is extruded from the process causing adjacent plasma membrane surfaces to become apposed. This results in a characteristic multilamellar structure. Apposition of cytoplasmic faces of plasma membrane forms the major dense line and a thinner intraperiod line results from apposed extracellular membrane faces. Some axons may be ensheathed by over 90 compacted bilayers (Lemke, 1986). The distances between nodes of Ranvier vary with axonal diameter, thinner axons have shorter internodes and generally axons with diameters of less than 1μm are not myelinated. Hence axon calibre seems to be an important element in determining myelination (Raine, 1984; Voyvodic, 1989). Figure 1.1 schematically demonstrates myelination of an axon.

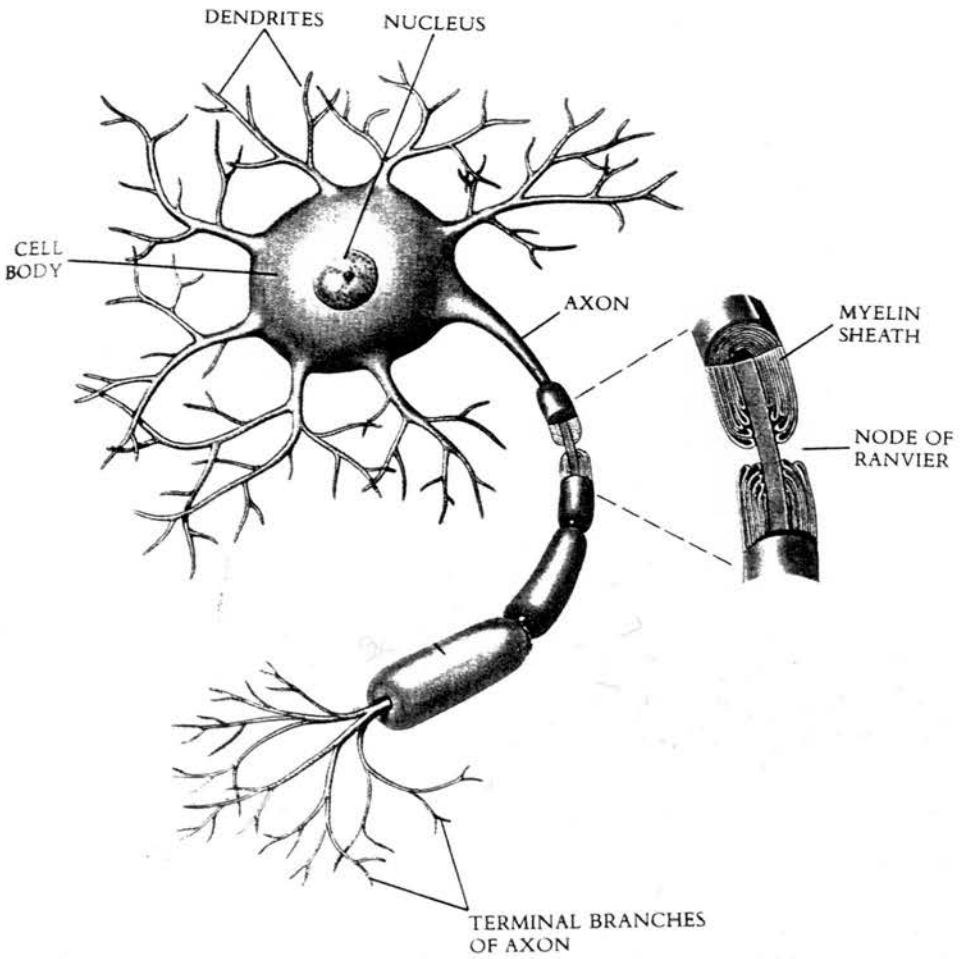
Asou and colleagues (Asou et al., 1994) observed by time lapse photography the initial events of this process of myelination. They showed that the axon is contacted several times by an oligodendrytic process which terminates with a lamellipodium that was seen to 'ruffle'. Eventually the terminal part of the process became much thicker and apparently anchored to the axon. This was followed by rotation of the process around the axon. Transitory junctional complexes between oligodendrocyte processes and axons were observed by Sims and colleagues (Sims et al. 1988). It is thought that these may stabilise and help to guide the myelin forming process around the perimeter of the axon. Their existence must be transitory to allow the inner spiralling oligodendrocyte process to proceed. Coated vesicles were also observed in the axonal cytoplasm or fused with the axolemma and may be responsible for the addition or removal of the specialised junctional

Figure 1.1 Schematic representation of myelination of a neuronal axon by oligodendrocytes.

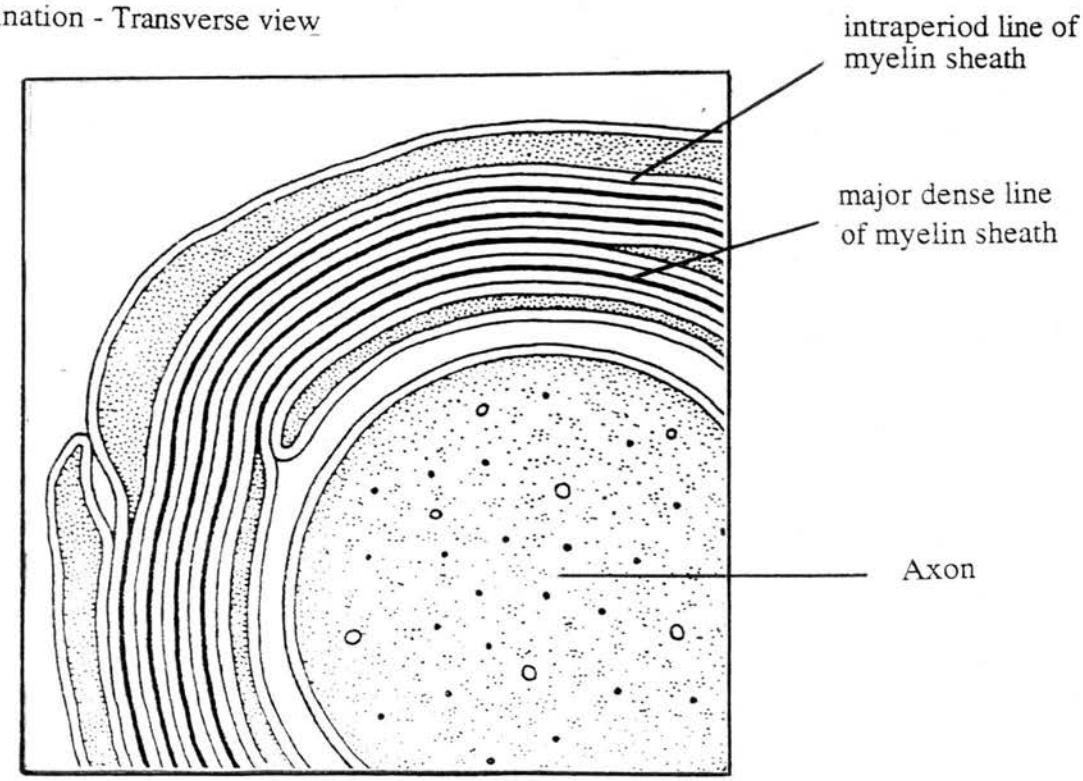
A. Longitudinal view demonstrating nodes of Ranvier which allow saltatory conduction of the impulse along the axon. Sheaths of myelin envelop the internodal regions. (Figure from Understanding Biology by Raven and Johnson. Published by Times Mirror Mosby)

B. Transverse view of an axon ensheathed by compact myelin demonstrating the major dense line formed by apposing cytoplasmic faces of myelin membrane and the thinner intraperiod line resulting from apposed extracellular membrane faces. (Figure from Morell, 1980)

A. Myelination - Longitudinal view



B. Myelination - Transverse view



membrane at the sites of adhesion between axon and myelinating process. (Sims et al., 1988). At least one full rotation of myelin membrane occurs before compaction commences (Wiggins et al., 1988). Under normal conditions the absence of compaction with advanced rotation is rare, except in cases of myelin disorders.

The myelination of nerves first appeared during evolution in Gnathostomata (Waehneldt et al., 1986). The Agnatha, which include hagfish and lamprey, posses glial cells which intimately surround nerve axons but myelin formation is not observed (Bullock et al., 1984). It is suggested that the acquisition of myelin forming glia occurred 400-450 million years ago during the lower Silurian period, unless the ancestral forms of hagfish already possessed this capacity in the upper Cambrian period (approximately 520 million years ago) but then later lost it (Waehneldt et al., 1986). Spiral myelination and the expression of characteristic myelin proteins appears to parallel vertebrate evolution since the complexity of the composition of proteins in CNS myelin increases significantly during the transition from fishes to tetrapods in keeping with the 'old evolutionary theme of simplicity to complexity' (Waehneldt et al., 1986) (see Figure 1.2).

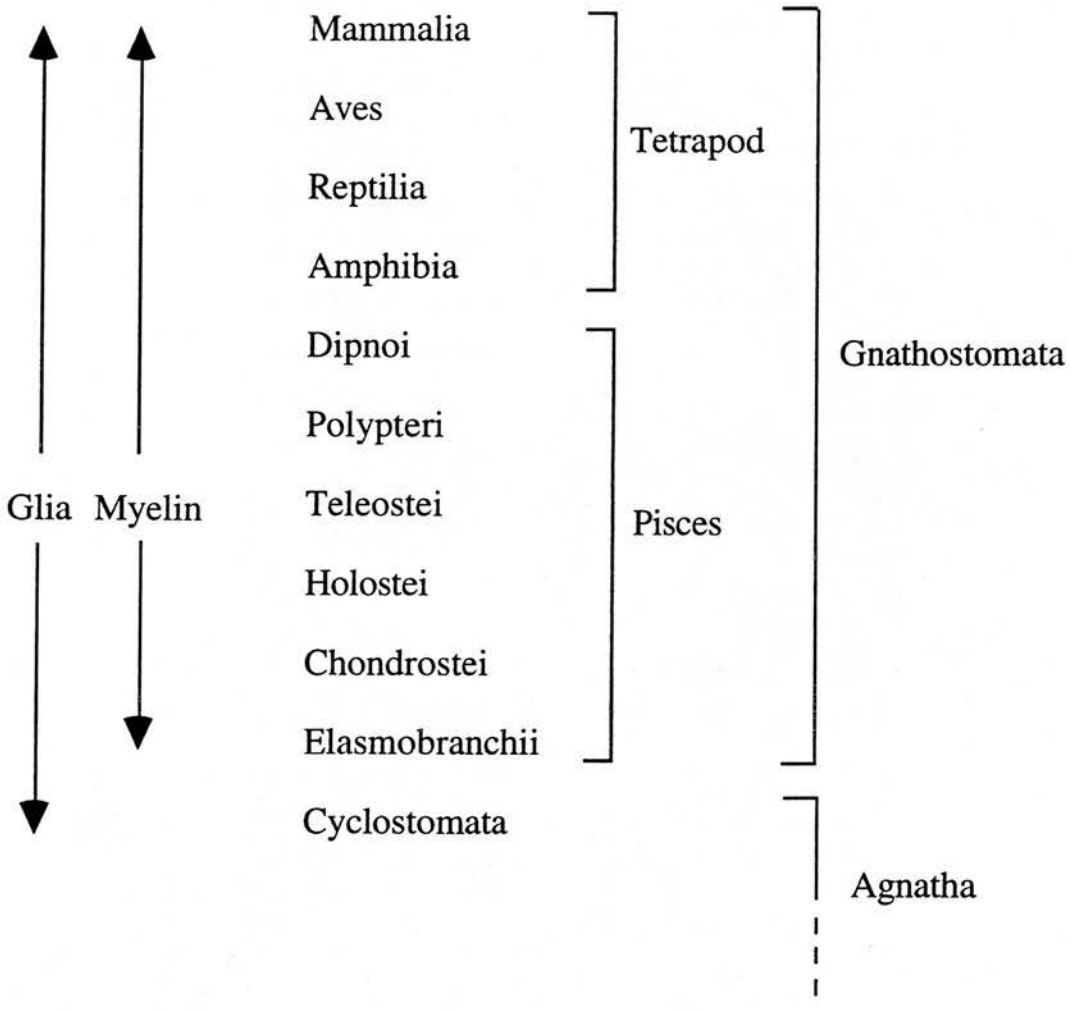
1.2 Components of CNS myelin

The process of myelination involves the production of myelin specific components by the oligodendrocyte. Like all biological membranes, myelin is composed of lipids and proteins. Approximately 70% of myelin (dry weight) consists of lipids, of which 32% are glycolipids, particularly galactocerebroside and sulphatide, its sulphated analogue, 26% is cholesterol and 42% are phospholipids (Pfeiffer et al., 1993). This high lipid content makes myelin highly suitable for its role as an electrical insulator of nerve fibres.

Galactocerebroside is synthesised by UDP-galactose ceramide galactosyltransferase (CGT). Bosio and colleagues (Bosio et al, 1996) have generated a null mutant mouse deficient in this enzyme which consequently completely lacks galactocerebroside and its sulfated analogue sulfatide in both the CNS and PNS myelin lipid bilayers. The velocity of nerve conduction in homozygous mutant mice is reduced to that of unmyelinated axons indicating a severely altered ion permeability of the lipid bilayer. The impaired insulator function is entirely due to the lack of the main glycolipids, galactocerebroside and sulfatide, and is not compensated for by other lipids. The severe dysmyelinosis causes body tremor, loss of locomotor activity and death.

Galactocerebroside and sulfatide are important in the structure and stability of the myelin lipid bilayer in three ways (Bosio et al., 1996). Firstly, they contribute

Figure 1.2 Diagram demonstrating the appearance of myelin in evolution.


Mammalia
Aves
Reptilia
Amphibia
Tetrapod
Dipnoi
Polypteri
Teleostei
Holostei
Chondrostei
Elasmobranchii
Pisces
Gnathostomata
Glia
Myelin
Cyclostomata
Agnatha

to the tight packing of the hydrophobic core domain, the fluidity of which is adjusted by the presence of cholesterol. Secondly, strong interactions with proteins such as myelin basic protein (MBP) and proteolipid protein (PLP) contribute to the tight compaction of the multilayer membrane system. Thirdly, a dense network of hydrogen bonds between cholesterol and other lipid components may contribute to the impermeabilty of the lipid bilayer to ions and therefore its insulation properties. Therefore in a mutant mouse lacking galactocerebroside and sulfatide, cholesterol and highly unsaturated phospholipids are the main constituents of the myelin membrane resulting in increased fluidity and ion permeability and therefore decreased insulating properties and saltatory conduction (Bosio et al., 1996).

Defects in other enzymes involved in lipid metabolism cause many disorders of the nervous system in humans. One group is the Adrenoleucodystrophies (ALD), some of which are X-linked recessive disorders. They are characterised by the accumulation of very-long-chain saturated fatty acids in all lipid containing tissues and body fluids and arise from defective very-long-chain fatty acyl-CoA synthetase activity in peroxisomes resulting in an accumulation of membrane like cytoplasmic inclusions in brain tissue (Wanders et al., 1988).

Proteins are less abundant than lipids in myelin. It has been suggested that approximately 30% of myelin (dry weight) consists of protein (Pfeiffer et al., 1993). The number of major proteins in myelin is relatively small. PLP comprises 50% of the protein mass, followed by MBP which comprises 30-40% in CNS myelin. 2´,3´-cyclic 3´-phosphohydrolase (CNPase) forms approximately 5% and myelin associated glycoprotein (MAG) forms less than 1% of myelin protein (Dubois-Dalcq et al., 1986; Pfeiffer et al., 1993). There are other minor proteins also present (e.g. myelin/oligodendrocyte glycoprotein (MOG)) and additional ones are continually being identified. Some of these proteins are believed to have specific roles in the formation and maintenance of the myelin sheath. Studies utilising mutant mice or knockouts have aided the elucidation of some of these roles. Described below are the best characterised proteins of CNS myelin with a discussion of their possible functions.

1.2.1 MBP

The myelin basic proteins (MBPs) are a family of small, exceptionally basic, membrane proteins localised at the major dense line of compact myelin (Oppenheim, 1996). They are thought to mediate compaction between adjacent cytoplasmic membrane surfaces. In the CNS MBP constitutes 30-40% of myelin protein but only 5-15% of PNS myelin protein (Lees and Brostoff, 1984). A single 32 kb gene for

MBP located on chromosome 18 (Roach et al., 1985) in both rats and mice encodes for at least six related proteins which are the products of alternative RNA splicing. Five of the protein variants have molecular weights of 14, 17, 18.5, 20 and 21.5 kD. A sixth form closely related to the 17 kD variant is indistinguishable on SDS polyacrylamide gels (SDS PAGE) and so avoided identification for some time (Lemke, 1988). In humans there are at least four different forms present. Therefore MBP is an example of a gene which is spliced differently in different species (Roth et al., 1987). Transcription of the gene occurs from a discrete promoter beginning during the first week of postnatal development, at a time coincidental with the onset of myelination (Zeller et al., 1985). Newly translated proteins are modified by phosphorylation (Lemke, 1988; Sutcliffe, 1987).

MBP is found diffusely distributed throughout the cytoplasm of oligodendrocytes in the early stages of myelination but then transfers to the cell processes and compact myelin as myelination progresses (Omlin et al., 1982; Zeller et al., 1985). Since MBPs exhibit a strong but non-specific affinity for membranes due to their high positive charge, mechanisms have evolved for transporting MBP mRNAs to regions where myelin compaction is occurring. This ensures the correct targeting of MBP polypeptides to their final destination. Ainger et al. observed distinct patterns of MBP mRNA movement in cultured oligodendrocytes following microinjection of labelled MBP mRNA (either with digoxygenin or a fluorophore) (Ainger et al., 1993). Immediately after injection the mRNAs formed into granules which associated with the cytoskeletal matrix and were not released by extraction with non ionic detergent. These granules then demonstrated a sustained directional movement along oligodendrocyte processes, closely associated with microtubules traversing the cytoplasm. At branch points there was oscillatory motion and at the cell periphery where the processes terminated the granules demonstrated random circular movements. It is suggested that microtubule based motor proteins e.g. kinesins are involved in the active transport of mRNAs along processes but microfilament based motors e.g. myosin I may be involved in localising the mRNAs at the cell periphery.

Recently Pedraza and colleagues investigated observations of MBP expression in the nuclei of immature oligodendrocytes (Pedraza et al., 1997). They show that MBP isoforms containing exon II, which are highly expressed in developing oligodendrocytes, are actively transported into the cell nucleus by a process which is temperature and energy dependent and may be regulated by phosphorylation. It is very unusual for a membrane protein to be targeted to the nucleus. Suggestions implicate that these MBPs have a role in initiating regulatory

mechanisms which coordinate the process of myelination. This hypothesis is supported by observations of the *shiverer* mutant mouse whose MBP gene is nonfunctional (Roach et al., 1985). The absence of MBP protein is associated with reduced expression of other unrelated myelin proteins (Ganser and Kirschner, 1980).

The *shiverer* mutant is phenotypically characterised by a generalised tremor first appearing at postnatal day 12 (P12) and thereafter worsening until premature death between P50 and P100 (Chernoff, 1981; Hogan and Greenfield, 1984). It is caused by the deletion of a large proportion of the MBP gene. The entire gene from a point approximately 12 kb downstream from exon 1 is missing (Roach et al., 1983). This MBP deletion seems to be the sole cause of the *shiverer* phenotype. Levels of MBP are reduced to less than 1% of wild type (Popko et al., 1987) and CNS myelin is largely absent. When myelin is present it is as abnormal whorls of cytoplasm filled membranes tightly compacted at intraperiod lines but uncompacted at major dense lines (Privat et al., 1979, Kirschner and Ganser, 1980). PNS myelin is less affected than CNS appearing almost structurally and functionally normal (Kirschner and Ganser, 1980). Insertion of the entire normal *MBP* gene into the genome of mutant mice causes the *shiverer* phenotype to be reversed in resultant transgenics expressing the introduced transgene (Readhead et al., 1987). The phenotype is also reversed in transgenics expressing a cDNA encoding the 14 kD MBP isoform (Kimura et al., 1989).

An independent autosomal recessive mutation, myelin deficient (*md*), is allelic to *shiverer* (Doolittle and Schweilkart, 1977; Hogan and Greenfield, 1984). The lifespan of these mutants is shorter than normal mice but longer than *shiverer* mice of the same genetic background. They also exhibit a classic shivering phenotype with tremors and convulsions which generally cause death at around 90 days. The CNS is severely hypomyelinated with approximately 2% of normal MBP RNA levels (Roch et al., 1986). With increasing age the amount of compact myelin increases in the CNS as do MBP protein levels but normal levels are never reached. A defect in the regulation of the MBP gene exists and these *md* mice also have several copies of the MBP gene, some with large rearrangements (Popko et al., 1987). Mice have been produced with graded amounts of myelin by the introduction of one or more copies of an MBP transgene which have facilitated studies of the role of MBP in myelination (Popko et al, 1987).

1.2.2 PLP

Proteolipid protein (PLP) is a major integral membrane protein of CNS myelin constituting about 50% of total protein. It is hydrophobic and has a possible

structural role in mediating interactions between opposing extracellular membrane surfaces in compact myelin. There is severe disruption of myelin compaction and a lack of the intraperiod line in *Plp* gene 'knock out' mice (Boison and Stoffel, 1994).

PLP is encoded by a single gene located on chromosome X (Willard and Riordan, 1985). Its sequence is highly conserved during evolution with 99% sequence homology in rats, cows and humans which suggests a strong structure/function relationship for PLP (Lemke, 1988; Sutcliffe, 1987). The PLP gene encodes several mRNA isoforms and thus it is known as a complex transcriptional unit. DM20 is a truncated form of PLP which arises by alternative splicing mechanisms. It differs from PLP by a deletion of 105 bp (Nave et al., 1987). In addition to these two main protein isoforms, each displays 5′ and 3′ heterogeneity (Kamholz et al., 1992; Milner et al., 1985). In the mouse there are two major transcriptional start sites. Transcriptional activation from the proximal site is favoured in the PNS in contrast to initiation from the distal site in oligodendrocytes. 3′ heterogeneity arises from the alternate use of three polyadenylation sites present in exon 7 giving rise to three PLP mRNAs of 3.2, 2.4 and 1.6 kb in the adult mouse.

The precise role of DM20 is as yet unknown but it is predominantly expressed during embryonic development. Experiments by Montague and Griffiths (Montague and Griffiths, 1997) recently have shown that between embryonic day 12 (E12) and E16 in mice DM20 is the predominant form, present at a ratio of 3:1. From E17 to postnatal day 1 (P1) there is a shift in the ratio with both protein isoforms being equally expressed. After P1 the ratio shifts again towards predominant expression of PLP at a ratio of 3:1. This relationship is maintained throughout adulthood and is therefore used as a recognised marker for post mitotic oligodendrocytes. Peak protein synthesis, in line with myelination, occurs several days after the basic proteins. PLP proteins are synthesised on membrane bound ribosomes in the oligodendrocyte cell body. This is followed by microtubule dependent delivery and insertion into the plasma membrane (Dubois-Dalcq et al., 1986). The protein has several membrane spanning domains but it does not utilise a cleaved amino-terminal signal peptide for insertion into the membrane as other integral membrane proteins do (Lemke, 1988; Sutcliffe, 1987). Signals present in internal domains of the molecule must be involved instead (Weimbs and Stoffel, 1992). Post-translationally the polypeptide backbone is acylated with fatty acids. Acylation occurs only as the protein begins entry into the myelin sheath and appears completed before it enters the compact myelin domains (Coleman et al., 1982; Townsend et al, 1982).

Mammalian DM20 has significant sequence similarity to related molecules in

primitive fish (DMα, β and γ) and in mice (M6a and b) (Yan et al., 1993). There is significant sequence identity of DMα to channel lining regions of glutamate and acetylcholine receptors so it has been proposed that DM20 may act as an adhesive pore in myelin membrane (Kitagawa et al., 1993). Adhesive pores composed of DM20 oligomers may line up, couple myelin membranes and form channels spanning the compact myelin lamellae. This may provide physiological communication between the extracellular space at the outer surface of the myelin sheath and the axonal membrane that is covered. A selectively porous sheath might also allow ion passage across the myelin lamellae. Putative transmembrane segments of PLP/DM20 are amphipathic as are corresponding regions in known channel proteins (Popot et al., 1991).

The *PLP* gene is the target of a host of mutations. Many single base missense changes are found to cluster within the putative membrane spanning region of the gene and contribute to multiple forms of Pelizaeus-Merzbacher disease (PMD) in humans. Partial and complete deletions, insertions, frame shifts and duplications also cause the disease (Hodes et al., 1993; Seitelberger, 1995). *Jimpy* is a defect in the PLP gene caused by a point mutation at the splice acceptor site immediately preceding the 5th exon (Nave et al., 1987). This mutant has a severely hypomyelinated central nervous system with degeneration and premature cell death of oligodendrocytes during development. *Rumpshaker* is a mouse mutant resulting from a point mutation within the M4 membrane domain (Fanarraga et al., 1992; Griffiths et al., 1990). This mutant also has severe hypomyelination which is more severe in the brain than the spinal cord and its optic nerve axons are completely unmyelinated. *Rumpshaker* mice, however have nearly normal lifespans unlike their *jimpy* cousins (Lemke, 1993). It has been suggested that PLP plays a role in oligodendrocyte development and survival as well as in the stabilisation of the myelin structure.

1.2.3 MAG

Myelin associated glycoprotein (MAG) is a minor transmembrane glycoprotein and is a member the immunoglobulin superfamily (Arquint et al., 1987). It is localised in oligodendrocytes to the periaxonal membrane immediately adjacent to the axon and is also found in non-compacted areas of the myelin sheath but is absent from compact myelin (Martini and Schachner, 1986). MAG has been detected in the processes of cultured oligodendrocytes at an age corresponding to P10 *in vivo*, a time before either MBP or PLP are expressed (Dubois-Dalcq et al., 1986). This protein is present at relatively low levels, constituting less than 1% of

myelin protein.

MAG is encoded by a single gene on mouse chromosome 7 (Barnton et al., 1987). It is a heavily glycosylated glycoprotein and has two polypeptide forms (72 and 67 kD) which are produced by alternative splicing mechanisms. Only the larger protein is expressed during the initial stages of myelination. The smaller protein is expressed in adults when myelination is almost complete and it has a shorter cytoplasmic domain than its larger counterpart (Frail and Braun, 1983). Small MAG is the major isoform in the adult CNS (Pedraza et al., 1991).

In vitro studies using anti-MAG antibodies or antisense strategies (Owens and Bunge, 1991) suggested that absence of functional MAG would result in severe defects of myelination. However mice with null mutations of the *Mag* gene develop apparently normal myelin sheaths of normal thickness and periodicity. In a few CNS fibres the cytoplasmic collar is not present or it contains abnormal organelles. Also the myelin present is redundant and the periaxonal space is enlarged. These defects are associated with a tremor and reduced motor activity (Li et al., 1994). Another study found no clinical phenotype in a *Mag* null mutant mouse (Montag et al., 1994) but CNS fibres showed a decreased periaxonal cytoplasmic collar and multiple myelin sheaths were present around some axons. The lack in phenotypical abnormalities, such as tremor, in these mice was ascribed to an increased expression of the cell adhesion molecule N-CAM (Montag et al, 1994).

MAG has features of a cell adhesion molecule such as the carbohydrate determinant HNK-1 which is common to molecules proposed to mediate cell-cell interactions e.g. NCAM and L1 (McGarry et al., 1983). MAG also contains the Arg-Gly-Asp tripeptide which mediates binding of many matrix adhesion molecules to their receptors (Salzer et al., 1987). For example, the cell receptors for fibronectin (Pytela et al., 1985b), vitronectin (Pytela et al., 1985a) and osteopontin (Oldberg et al., 1986) have been shown to interact with Arg-Gly-Asp sequences on these extracellular matrix proteins. MAG has been shown to mediate neuron-oligodendrocyte and oligodendrocyte-oligodendrocyte adhesion *in vitro* which is subsequently blocked by an anti MAG antibody which recognises an extracellularly localised epitope (Poltorak et al., 1987). Of the two isoforms only the larger protein is phosphorylated, primarily at serine residues, and it is suggested that this has significance in the regulation of oligodendrocyte-neuron interactions (Edwards et al., 1988b). Thus evidence suggest that MAG may have a role to play in coordinating the recognition and interaction of oligodendrocyte processes with axons and in preventing interactions with existing myelin.

1.2.4 CNPase

2′,3′-cyclic nucleotide 3′-phosphohydrolase (CNPase) enzymatic activity was first demonstrated in bovine spleen (Whitfield et al., 1955) and pancreas (Davis and Allen, 1956). It hydrolyses 2′,3′-cyclic nucleotides to give 2′-nucleotides and can be induced by cyclic AMP (McMorris, 1983). It exists as a dimer with two catalytically active enzyme forms (Mueller et al., 1981) produced by alternative splicing of mRNAs (Bernier et al., 1987). It has been detected in a variety of tissues including neuronal cell lines, cultured astrocytes, adrenal medulla, rat liver mitochondria and serum but its high specific activity in myelin of both the peripheral and central nervous systems has resulted in its use as a biochemical marker for myelin and more specifically for the myelin forming glial cells since by comparison its activity is extremely low in astrocytes (Kurihara and Tsukada, 1967; Podulso and Norton, 1972). CNPase is found throughout the developing oligodendrocyte cell body but is more concentrated at the cell periphery adjacent to the plasma membrane and in elongated oligodendrocyte processes (Braun et al., 1988). In myelin CNPase is excluded from compact lamellar domains but is present within the cytoplasmic channels that traverse the sheath (Trapp et al., 1988; Brunner et al., 1989). It is an accumulating component of myelin appearing early in oligodendrocyte development followed later by MAG, MBP and PLP expression (McMorris, 1983; Vogel and Thompson, 1988). Translation occurs on free ribosomes (Gillespie et al., 1986; Karin and Waehneldt, 1985) and the amount of translatable RNA increases with myelination. Both isoforms of the enzyme are also developmentally regulated in parallel (Edwards and Braun, 1988a).

1.2.5 MOG, MOSP, MOBP and OMgp

Myelin/oligodendrocyte glycoprotein (MOG) is a unique member of the immunoglobulin superfamily expressed specifically in the CNS (Gardinier et al., 1992). Immunoelectron microscopy has localised this protein to the processes of oligodendrocytes and the external surface of the myelin sheath in adult rats (Linington et al., 1988). MOG expression parallels other myelin proteins peaking at P15-25 in rats, a period of active myelination but expression lags two days behind MBP possibly suggesting a function in completion and maintenance of the myelin sheath (Gardinier et al., 1992). MOG has been shown to be a primary target autoantigen in autoimmune encephalomyelitis in Lewis rats and so may act as an autoantigen in the pathogenesis of some demyelinating diseases such as multiple sclerosis in humans (Adelmann et al., 1995).

Myelin/oligodendrocyte specific protein (MOSP) is specifically expressed in

CNS myelin and oligodendrocytes of higher vertebrates and is recognised by the monoclonal antibody CE1 (Dyer et al., 1991). MOSP initially appears at P4-5 about 1-2 days after the appearance of galactocerebroside and sulfatide (Mu and Dyer, 1994). This coincides with the time when oligodendrocytes are elaborating processes and just beginning to form membrane sheets. It is a 48 kD protein which remains on the surface of cultured oligodendrocytes but becomes associated with cytoplasmic microtubules following antibody binding (Dyer et al., 1991). This suggests a role in membrane/cytoskeleton interactions during the extension of growing processes and the formation of the myelin sheath. Its expression on the surface of cultured oligodendrocytes also suggest that it may serve as an antigenic target in demyelinating diseases (Dyer et al., 1991).

Myelin-associated/oligodendrocytic basic protein (MOBP) was identified by a subtractive cDNA screening approach (Schaeren-Wiemers et al., 1995; Yamamoto et al., 1994). It has several isoforms produced by alternative splicing which are identical at their N-termini but differ at their C-termini. MOBP expression is exclusive to the CNS (Yamamoto et al., 1994). Expression in rat optic nerve oligodendrocytes occurs later than MBP and PLP but coincides exactly with the onset of myelin compaction suggesting a possible role in the last stages of myelination and maintenance of the myelin sheath (Holz et al, 1996).

Oligodendrocyte myelin glycoprotein (OMgp) is a membrane protein specific to oligodendrocytes and CNS myelin (Mikol and Stefansson, 1988). The gene is located to chromosome 17 in humans (Mikol et al., 1990b). Its function is unknown but it is a member of a protein family characterised by leucine-rich repeats (Mikol et al., 1990a).

1.3 Are other cell types involved in myelination?

The other major macroglial cell type is the astrocyte characterised by intermediate filaments which are largely composed of polymers of GFAP (glial fibrilliary acidic protein). GFAP is a specific marker for these cells in the CNS (Bignami et al., 1972). Astrocytes are found ubiquitously throughout the CNS and are thought to be the most abundant glial cell type. They exhibit different properties depending on anatomical location. In grey matter protoplasmic astrocytes have fine delicate processes but display little immunoreactivity for GFAP. In white matter fibrous astrocytes have thicker processes with low but detectable immunoreactivity for GFAP (Lee and Brosnan, 1997).

Astrocytes have a number of functions *in vivo* including the formation of physical barriers within the nervous system. Cells resembling fibrous astrocytes

contribute to glial limiting membranes (glial limitans) (Miller and Raff, 1984). The processes of specialised astrocytes put feet on blood vessels forming the perivascular sheath and can induce endothelial cells to form tight junctions (Janzer and Raff, 1987). This suggests that glial cells are responsible for inducing the capillary and venule endothelial cells in the CNS to form the blood brain barrier. Astrocytes are also part of the physical barrier separating the CNS and the pia-arachnoid membrane, the CNS-CSF (cerebral spinal fluid) barrier (Lee and Brosnan, 1997).

Fibrous astrocytes have been observed to interact directly with neurons by extending processes to the exposed regions of axon between myelin sheaths at the nodes of Ranvier where saltatory conduction occurs. Their role is possibly one of nourishing the adjacent axon and stabilisation of local ion concentrations (Ffrench-Constant and Raff, 1986). Astrocytes are known to secrete growth factors which act on developing oligodendrocytes (Noble and Murray, 1984). There is also some indirect evidence that astrocytes are responsible for forming glial scarring in CNS white matter. Following injury a glial scar forms which is composed of intermediate filament bundles generated by increased levels of GFAP, vimentin and nestin. The intermediate filament network at the scar area is very compact and it has been proposed that the glial scar acts as a physical barrier to neurite outgrowth (Hatten et al., 1991). Nestin, like GFAP, is an intermediate filament protein which forms an easily recognisable cytoskeletal network, a general characteristic of intermediate filaments (Lendahl et al., 1990). The protein is predominantly present in CNS progenitor cells and expression is downregulated in the adult nervous system (Dahlstrand et al., 1995). In addition to normal expression during development, the nestin gene can be reactivated in situations of stress or induced proliferation (Dahlstrand et al., 1992). After injury to the nervous system nestin is induced in the CNS. Nestin expression is strikingly and rapidly upregulated in astrocytes of an injury area and its induction lasts for at least 13 months leaving a longlasting molecular imprint of the damage (Frisen et al., 1995). Antisense GFAP RNA has been shown to inhibit GFAP synthesis in astrocytes and so this technique may be useful for regulating the astrogliosis immediately following CNS injury by delaying the gliotic reaction and formation of the physical barrier, thus enabling neurons and oligodendrocytes to re-establish a functional environment (Yu et al., 1991).

Two types of $GFAP^+$ astrocytes have been identified in cultures of developing rat optic nerve based on morphology, growth characteristics and their labelling with ligands. Type 1 astrocytes have a fibroblast like morphology, proliferate in culture and do not bind tetanus toxin or the A2B5 antibody (Raff et al., 1983). Type 2 astrocytes have a process bearing morphology similar to

oligodendrocytes, divide infrequently in culture and bind tetanus toxin and the A2B5 antibody (Raff et al., 1983). Expression of both A2B5 and GFAP is a useful marker in this culture system for type 2 astrocytes, which also share a progenitor cell stage with oligodendrocytes *in vitro* (see 1.4). In developmental studies, these markers were used to identify astrocyte subtype in cultures from optic nerves taken throughout development. Miller et al. observed that only type 1 astrocytes ($GFAP^+/A2B5^-$) were present at the time equivalent to birth and they reached stable levels by P15. Type 2 astrocytes ($GFAP^+/A2B5^+$) did not appear until the second postnatal week after P15, corresponding to a time after oligodendrocytes have begun myelination. Type 2 astrocytes contributed to more than 65% of astrocytes in cultures of adult nerve (Miller et al., 1985). A third type of astrocyte has been identified in cultures of striatum and spinal cord, which express GFAP and the GD3 ganglioside (Vaysse and Goldman, 1992). This cell type is clonally distinct from type 1 and type 2 astrocytes. These lineage distinctions described may have little applicability to astrocytes throughout the CNS *in vivo* since to date there are no lineage specific subtype markers which encompass both *in vivo* and *in vitro* astrocytes. Astrocytes are extremely heterogeneous not only when comparing astrocytes from different brain regions but also when comparing cells from the same brain region (Lee and Brosnan, 1997).

During development a caudal to rostral gradient of GFAP expression by astrocytes can be followed which correlates with overall brain maturation. GFAP mRNA is first seen at E16 in the glial limitans of the ventral hind brain but peak expression at P15 is localised to the deep white matter tracts of the cerebellum, corpus callosum and certain hippocampal tracts. This is reduced in adults (Landry et al., 1990). The expression of GFAP mRNA in the developing brain is consistent with a pattern of at least two waves of astrocyte development. The differentiation of interfascicular glia in both white and grey matter occurs first followed by the appearance of astrocytes in white matter tracts once myelination has been initiated (Landry et al., 1990). A similar pattern of two waves of astrocyte development was observed by Miller et al in the developing rat optic nerve, as described above. The correlation of astrocyte development with the onset of myelination implicates a role by astrocytes in this process during development of nervous system.

1.4 A closer look at myelination

Myelination at first glance appears deceptively simple. Apparently few major components contribute to the elegant structure of the myelin sheath which so effectively fulfils its role as an electrical insulator, thus enabling the body to function

in a coordinated manner. Yet the details of the process are still extremely poorly understood and many workers continue to explore the gaps of knowledge striving to piece together a full picture of understanding.

1.4.1 What causes an oligodendrocyte to extend processes which will myelinate neuronal axons?

Mature multipolar oligodendrocytes are located in brain white matter as rows of myelinating cells between nerve fibres. In grey matter they are either associated with myelinated axons or exist as non-myelinating satellite cells. They are characterised by their expression of galactocerebrosides (GC) and the lack of vimentin or any other class of intermediate filaments. These mature cells develop from small, rounded, bipolar progenitor cells which express vimentin in their cytoplasm and gangliosides on their cell surface recognised by the A2B5 monoclonal antibody. Such a progenitor cell can be isolated *in vitro* and will differentiate in the presence of foetal calf serum (FCS) into a GFAP expressing type 2 astrocyte ($A2B5^+$, $GFAP^+$) or into an oligodendrocyte ($A2B5^-$, GC^+) when FCS is not present. This bipotential progenitor cell therefore became known as the oligodendrocyte-type 2 astrocyte (0-2A) progenitor (Raff et al., 1983) and *in vitro* studies on it have consequently provided many insights into how the development of oligodendrocytes is regulated. *In vivo* the tetrasialogangliosides which bind the A2B5 antibody are also present on neurons (Eisenbarth et al., 1979), but the optic nerve which has been described as the simplest part of the CNS does not contain neuronal cell bodies and so A2B5 is a useful marker for these progenitor cells present (Miller et al., 1985).

Previously there has been some debate about whether type 2 astrocytes develop from 0-2A progenitor cells *in vivo*, although it is now generally thought to be unlikely. The optic nerves of newts posses a single type of glial cell which functions as both an astrocyte, with glial filaments and forming a glial limiting membrane, as well as an oligodendrocyte which myelinates the axons. In the spinal cord of the newt, however, these two functions are performed by different cell types. This suggests that there is a possible phylogenetic relationship between type 2 astrocytes and oligodendrocytes (Raff et al., 1983). However since there is no conclusive evidence that 0-2A cells give rise to type 2 astrocytes *in vivo*, the 0-2A cells are now referred to as oligodendrocyte progenitors *in vivo*.

Cell lines of glial progenitor cells, however, do appear able to generate both oligodendrocytes and astrocytes *in vivo* as well as in vitro in response to their environment. Following the transplantation of cells of the CG4 cell line into areas of

adult rat spinal cord white matter which had been permanently depleted of glial cells by local X-irradiation and/or injection with 0.1% ethidium bromide, both myelin forming oligodendrocytes and GFAP$^+$ astrocytes were identified within the lesion (Franklin et al., 1995). The CG4 cell line was obtained from cultures of 0-2A progenitor cells which had been maintained in the presence of mitogens secreted by neuronal B104 cell line (Louis et al., 1992). The origin of the glial cells within the spinal cord lesion was confirmed by retroviral incorporation of the lacZ marker gene and hence demonstrated that CG4 cells have the potential to differentiate along both glial pathways in an *in vivo* environment (Franklin et al., 1995). This indicates that clonal glial progenitor (0-2A) cell lines are able to manifest differentiation bipotentiality within a pathological environment that does not appear to occur during normal development (Skoff, 1990). This has obvious implications in the repair of damaged CNS.

1.4.2 How do 0-2A progenitor cells decide which glial pathway to take?

Progenitor cells are able to switch from the astrocyte pathway to the oligodendrocyte pathway after one or two days but not after three days in culture, so cell division and hence DNA replication is not a requirement for 0-2A progenitors to choose which glial pathway to differentiate along (Temple and Raff, 1985). Since type 2 astrocytes occur in culture in the presence of FCS it seems that some factor mimicked by FCS induces this choice of pathway and the oligodendrocyte pathway must therefore be a constitutive one. There is evidence to suggest that ciliary neurotrophic factor (CNTF) released by type 1 astrocytes has such an inducing effect on type 2 astrocytes (Raff, 1989).

1.4.3 Markers of the oligodendrocyte lineage.

The expression of myelin constituents and the process of myelination both occur as a consequence of the differentiation of 0-2A progenitors into oligodendrocytes. Antibodies raised against the various myelin proteins are used to follow their sequential appearance and hence the stages of oligodendrocyte development. The monoclonal antibodies 01 and 04 recognise developmentally regulated cell surface antigens which are specific to cells of the oligodendrocyte lineage. 04 is a marker for sulfatide and seminolipid and 01 recognises galactocerebrosides (Sommer and Schachner, 1981). The ordered and partially overlapping expression of A2B5, 04 and GC/01 markers distinguishes 3 consecutive phenotypically defined stages of the oligodendrocyte lineage in vitro. 1) Bipolar A2B5$^+$, 04$^-$, GC/01$^-$ glial precursor cell, the 0-2A progenitor. 2) multipolar A2B5$^+$,

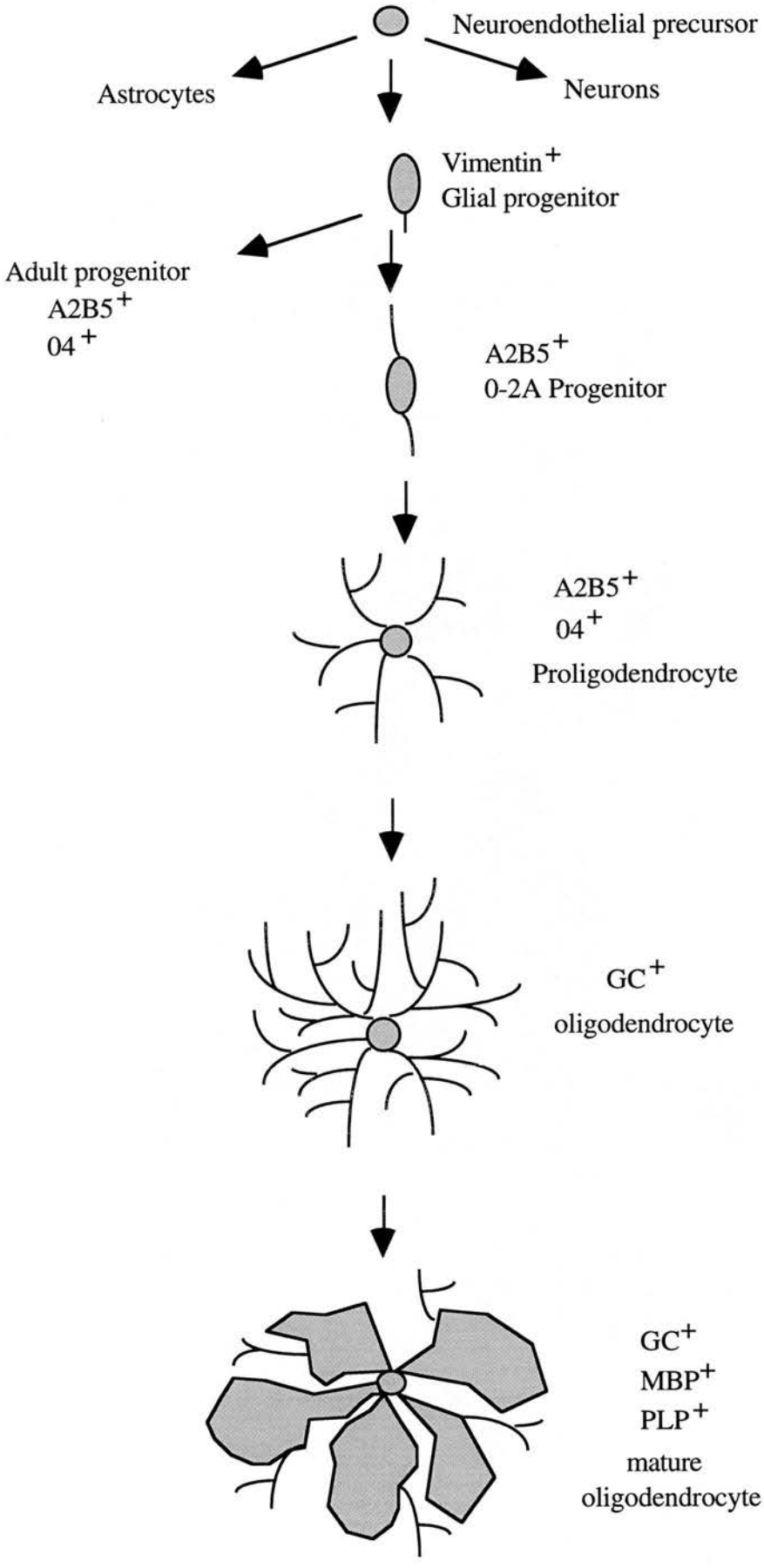
04^+, $GC/01^-$ progenitor. 3) complex process bearing 04^+, $GC/01^+$ oligodendrocyte (Dubois-Dalcq, 1987) which goes on to express the myelin proteins, CNP, MOSP, MBP, MAG, PLP and MOBP as it begins to produce myelin membrane (See Figure 1.3).

Other serological markers for cells of the oligodendrocyte lineage include the monoclonal antibody A007 which has a developmental expression pattern identical to that for 04 (Knapp, 1991). Both antibodies recognise the proligodendroblast antigen (POA) which is expressed by A007 and 04 positive oligodendrocyte progenitors before the appearance of sulfatide and galactocerebroside. It seems likely therefore that both antibodies either recognise the same epitope, or different epitopes on the same molecule, or two epitopes of two different molecules expressed at the same time (Bansal et al., 1992). The monoclonal antibody 2B10 labels a cell surface molecule specifically expressed by cells of the oligodendrocyte lineage, first appearing as early as embryonic day 14 in rat spinal cord. Its abundance decreases during myelination but remains steady in adulthood. This molecule is not, however, a myelin-associated protein since it is not detected in purified myelin fractions (Zhou et al., 1995). The monoclonal antibody Rip selectively stains mature oligodendrocytes, their processes and myelin in the adult CNS. It is exceptional in that it allows the long cytoplasmic processes to be traced back to a parent oligodendroglia somata (Friedman et al., 1989). When Rip is used in double labelling experiments with an antibody to carbonic anhydrase II (CAII), two biochemically and morphologically distinct populations of oligodendrocytes are identified. Rip^+, $CAII^+$ oligodendrocytes support numerous myelin sheaths for small diameter axons, while Rip^+, $CAII^-$ oligodendrocytes support fewer myelin sheaths for large diameter axons, hence there are two types of oligodendrocytes myelinating large and small diameter fibres (Butt et al., 1995).

1.4.4 Oligodendrocyte progenitor migration.

Oligodendrocyte progenitor cells are highly motile as demonstrated by time lapse cinematography of progenitor cells in the rat optic nerve (Small et al., 1987). Small et al. deduced that these progenitors are the only cells of the oligodendrocyte lineage capable of migration. They were observed moving in the direction of their bipolar processes and on division the two new cells migrated in opposite directions. On differentiation their migration ceased. During development of the rat optic nerve oligodendrocyte progenitor cells first appeared only after axons had reached the chiasm at approximately embryonic day 15 (E15). At E17 they were distributed throughout the nerve in a highly graded fashion with less than 0.4% in the retinal

Figure 1.3 Schematic representation of the rodent oligodendrocyte lineage *in vitro*. The sequential appearance of stage-specific antigens is indicated as the cell morphology changes resulting from differentiation. During differentiation the bipolar progenitor cell extends many processes which become highly branched and eventually produce sheets of myelin membrane which would normally envelop axonal segments. In culture flattened sheets of myelin membrane are apparent in mature oligodendrocytes.


Neuroendothelial precursor
Astrocytes
Neurons
Vimentin+
Glial progenitor
Adult progenitor
A2B5+
04+
A2B5+
0-2A Progenitor
A2B5+
04+
Proligodendrocyte
GC+
oligodendrocyte
GC+
MBP+
PLP+
mature
oligodendrocyte

segment, 2.4% in the middle segment and 6.5% in the chiasmal segment of the optic chiasm. By birth a steep gradient still existed but now 18% of progenitors occurred in the retinal segment, 31% in the middle and 51% in the chiasmal segment. This gradient diminished during the first nine days after birth. Small and colleagues deduced that the oligodendrocyte progenitor cells migrated into the optic nerve from the brain guided by the axons. The neuroepithelial cells forming the optic stalk therefore only give rise to astrocytes (Small et al., 1987). It is this ability of rapid movement over long distances by these small motile progenitor cells which ensures that all axons that need to be myelinated are catered for.

1.4.5 What controls the timing of oligodendrocyte cell development?

It is possible to culture oligodendrocytes in vitro which express myelin proteins e.g. MBP and PLP without the presence of neurons or other cell types. This suggests therefore that development of these cells must partly be intrinsic (Dubois-Dalcq et al., 1986; Temple and Raff, 1985; Knapp et al., 1987; Yim et al., 1986). However, there are also many factors which contribute to the regulation of oligodendrocyte development.

The sequential appearance of type 1 astrocytes ($A2B5^-$, $GFAP^+$) first appearing in the rat optic nerve at E16, oligodendrocytes (GC^+) appearing at birth and type 2 astrocytes ($A2B5^+$, $GFAP^+$) between P7 and P10 (Miller et al., 1985) suggest that astrocytes may be part of these regulatory mechanisms. Type 1 astrocytes have been shown to promote progenitor motility and inhibit premature differentiation in culture. The effect is produced when 0-2A cells are cultured in medium which has been conditioned by astrocytes but does not occur when the astrocytes are previously treated with antibodies against platelet derived growth factor (PDGF) (Raff et al., 1988). PDGF reproduces the effect on 0-2A cells when it replaces astrocyte conditioned medium and again the effect is blocked by an antibody against PDGF (Noble et al., 1988). Cultures of purified type 1 astrocytes have been shown to secrete PDGF as well as synthesise PDGF mRNA (Richardson et al., 1988). When astrocyte conditioned medium is fractionated by gel filtration the mitogenic activity on 0-2A cells is found in the same fraction as radiolabelled PDGF (Richardson et al., 1988) and since PDGF has also been shown to be present in the developing rat optic nerve (Pringle et al., 1989), evidence implicates PDGF as a molecule promoting progenitor motility and proliferation.

It has been hypothesised that PDGF derived from type 1 astrocytes drives an intrinsic clock which times oligodendrocyte development by counting the number of times a progenitor cell divides before spontaneously differentiating into a multipolar

oligodendrocyte (Raff et al., 1988; Raff et al., 1985). Most progenitor cells have similar cell cycle times dividing every 1-2 days in culture but their proliferative capacity varies from cell to cell. In an experiment when progenitor cells were isolated from P1 and P7 rat optic nerve, clone sizes ranging from 2-150 cells were produced. Separated sister cells always underwent a similar number of cell divisions before differentiation but the number of divisions varied from pair to pair (Temple and Raff, 1986). Hence the mitotic clock hypothesis suggests that there is a fixed maximum number of cell divisions that a progenitor cell can undergo before differentiation but this number varies between cells so that at any one time during development there are cells present at different developmental stages.

1.4.6 How is the mitotic clock timed?

In development the thyroid gland becomes active and produces thyroid hormone at around the time of birth coinciding with the first appearance of oligodendrocytes (Dussault and Ruel, 1987; Miller et al., 1985). Thyroid hormone is known to influence many types of precursor cell (Barres et al., 1994). In culture the number of divisions a progenitor cell undergoes before differentiating decreases with increasing levels of thyroid hormone (Barres et al., 1994). This observation appears to be consistent with the mitotic clock hypothesis. Glucocorticoids and retinoic acid have similar effects. The receptors for all these hormones, when complexed with their substrates, are able to inhibit the activity of the AP-1 transcription factors and therefore arrest cell division (Diamond et al., 1990; Nicholson et al., 1990). These transcription factors are formed by the dimerisation of members of the Jun and Fos protein families and help to mediate the proliferative response to growth factors e.g. PDGF in many cell types (Ransone and Verma, 1990; Angel and Karin, 1991). The intrinsic clock may therefore operate by counting the number of cell divisions promoted by PDGF which are permissible before AP-1 activity is decreased to a minimum level. As hormone levels increase, the hormone receptors inhibit AP-1 activity by either decreasing the concentrations of c-fos or c-jun, causing a change in their dimer partners or covalently modifying these proteins. Once AP-1 activity has fallen below a minimum level further proliferation is prevented and differentiation may proceed by default (Barres et al., 1994).

1.4.7 Effects of glutamate and cAMP

Other molecules also exert their effects on oligodendrocyte regulation through transcription factors at the level of gene transcription. Glutamate induces a rapid and transient expression of the c-fos oncogene in oligodendrocyte progenitor

cells through AMPA/kainate receptors and promotes progenitor cell proliferation (Liu and Almazan, 1995). This expression is also dependent on extracellular calcium influx and downstream activation of phorbal ester-sensitive protein kinase C (Liu and Almazan, 1995). Consequently glutamate plays a role in modulating the growth and differentiation of oligodendrocytes in the CNS. 3′, 5′-cyclic AMP (cAMP) is another molecule able to regulate oligodendrocyte development (Raible and McMorris, 1993). Analogues of this molecule inhibit proliferation and accelerate the rate of differentiation by progenitor cells in mixed glial cultures which have been established from 1 day old rat cerebrum (Raible and McMorris, 1989, 1990). This suggests that cAMP also increases the rate of induction of the expression of myelin components. The inhibition of proliferation and the promotion of differentiation by cAMP can be uncoupled, hence these two processes are regulated independently by cAMP (Raible and McMorris, 1993).

1.4.8 Production and deposition of myelin proteins

Not only is the differentiation of progenitor cells into oligodendrocytes carefully regulated but also, it seems, the production of myelin proteins once the cells have committed to this change in morphology. Monge and colleagues observed that in newly formed oligodendrocytes, just after differentiation, GC expression is followed by MBP and then PLP yet in the myelin sheaths deposition of MBP is followed by PLP and finally by GC (Monge et al., 1986). Therefore expression of these proteins within myelin appears to occur as a second wave following differentiation and shortly before oligodendrocytes begin the process of myelination (Monge et al., 1986). There is also a difference in timing between differentiation and myelination by oligodendrocytes in the optic nerve (Colello et al., 1995). Progenitor cells present throughout the length of the rat optic nerve at postnatal day two (P2) mature into GC^+ oligodendrocytes in a chiasm to eye progression, with the oligodendrocytes near the chiasm expressing MBP and PLP three days before those near the eye (Colello et al., 1995). However, Colello and colleagues also reported that the axonal segments near the eye are ensheathed with myelin before those near the chiasm and that the signalling for the onset of myelination is independent of the electrical activity of the axon but correlates with increased axonal diameter (Colello et al., 1995). They also noted that the proportions of axons near the eye were larger than axons near the chiasm and suggested that a level of axonal maturity is necessary before the potential expression of an as yet unknown factor which promotes myelination is possible. A gradient of this factor would therefore occur in an eye to chiasm direction thus promoting myelination of axons with a minimum diameter.

This would be similar to what has been found for PNS axons where axon calibre appears to be a crucial determinant of whether an axon becomes myelinated (Voyvodic, 1989). Therefore it seems that the processes of cell differentiation and the production of myelin by an oligodendrocyte can be uncoupled and regulated independently according to the requirements of the environment.

Steroid hormones effect myelin synthesis by differentiated oligodendrocytes. In culture hydrocortisone increases PLP mRNA expression by oligodendrocytes as well as the synthesis of cerebrosides, both of which are components of myelin (Podulso et al., 1990). Also the metabolising enzymes for ketone bodies, which are precursors of lipid synthesis, are induced by hydrocortisone (Robinson and Williamson, 1980). Lipids are an important component of the myelin sheath comprising 70% of its dry weight (Pfeiffer et al., 1993).

1.4.9. The role of the cytoskeleton.

The dramatic changes in cell morphology during differentiation and myelination by an oligodendrocyte are also dependent on the cytoskeleton which interacts with the plasma membrane to regulate shape and movement. Except for a transient expression of vimentin oligodendrocytes appear to be devoid of intermediate filaments (Pfeiffer et al., 1993) but their cytoskeleton is rich in microtubules (polymers of tubulin) and microfilaments (polymers of actin). Microtubules are located predominantly in the cell body and major processes of cultured oligodendrocytes while microfilaments are found enriched in the multiple fine processes at the cell periphery (Wilson and Brophy, 1989). Both play an important part in the intracellular sorting and transport of components as the changes in cellular architecture occur. When a cytoskeletal matrix is purified from rat CNS myelin by non-ionic detergent extraction, components specific to myelin such as MBP and CNP are found associated with the cytoskeletal preparations (Gillespie et al., 1989; Pereyra et al., 1988). This suggests the importance of such interactions during myelination and the maintenance of the mature myelin sheath.

Gelsolin is an ubiquitous actin binding and microfilament severing protein predominantly expressed in the CNS by oligodendrocytes (Lena et al., 1994; Tanaka and Sobue, 1994). There are two major forms of gelsolin, a cytoplasmic and a secretory form (Kwiatkowski et al., 1986). Recently an alternatively spliced form of the cytoplasmic gelsolin has been identified which appears to be exclusively expressed by oligodendrocytes in the CNS (Rogers et al., 1997). Actin-binding proteins are probably important in the extension of myelinating processes by oligodendrocytes.

Microtubule-associated proteins (MAPs) are important in regulating the dynamics and structure of microtubules (Mandelkow and Mandelkow, 1995) and are enriched in the CNS. The major types are either structural (MAP1, MAP2, MAP4 and MAP-tau) or are microtubule-based motors such as the kinesins which, as already mentioned, may be involved in the transport of MBP mRNAs to the oligodendrocyte periphery. MAP1B is present in oligodendrocytes but expression is absent from progenitor cells ($A2B5^+$, 04^-). It first appears in proligodendrocytes ($A2B5^+$, 04^+) and precedes the development of the mature oligodendrocyte phenotype suggesting that interactions between microtubules and MAP1B might have a role in the formation and stabilisation of developing myelin forming processes (Vouyiouklis and Brophy, 1993). This hypothesis is supported by the fact that anti-sense oligonucleotides to MAP1B mRNA have a destabilising effect on oligodendrocyte morphology in culture (Rogers et al., 1997).

MAP4 is ubiquitous but abundantly expressed in brain oligodendrocytes (Vouyiouklis and Brophy, 1995). It is expressed at all developmental stages of oligodendrocytes but does not colocalise with microtubules to the same extent as MAP1B in mature cells (Vouyiouklis and Brophy, 1995). Therefore it probably does not have a microtubule stabilising role as proposed for MAP1B. However since it is ubiquitous and upregulated during oligodendrocyte differentiation at a time when oligodendrocyte progenitors drop out of mitosis, it has been hypothesised that MAP4 is involved in regulating microtubule dynamics during the cell cycle (Olmsted et al., 1989).

MAP2 has two high molecular weight protein forms, MAP2a and MAP2b of approximately 199 kD and a smaller isoform, MAP2c, of 42 kD (Riederer and Matus, 1985; Kindler et al., 1990). These variants arise from a common gene by alternative splicing mechanisms (Papandrikopoulou et al., 1989). MAP2c is abundant at birth but is downregulated when the high molecular weight MAP2 becomes detectable, so is often referred to as the embryonic form of MAP2 (Reiderer and Matus, 1985). MAP-tau and the high molecular weight form of MAP2 are not present in cultured oligodendrocytes but MAP-tau has been detected in astrocytes (Vouyiouklis and Brophy, 1995). A fourth isoform of MAP2 has recently been identified which is similar to MAP2c but includes an additional repeat within the microtubule-binding site. It is therefore known as 4-repeat MAP2c or MAP2d (Vouyiouklis and Brophy, 1995; Doll et al., 1993). Although the specific functions of the various MAPs in oligodendrocytes are still unknown, their localisation patterns and expression during oligodendrocyte development suggest that they have distinct roles in the regulation of the microtubule network during

differentiation of oligodendrocytes.

1.4.10 How are the number of oligodendrocytes matched to the number of axonal segments to be myelinated?

The survival of newly formed and mature oligodendrocytes is also regulated by many factors. Between P8 and P12 at least 10,000 newly formed oligodendrocytes, approximately 50% of those generated each day, normally die in the optic nerve daily. They apparently die as a result of competition for survival factors which act to inhibit an intrinsic suicide program (Raff, 1992). In culture PDGF is a survival factor for newly formed oligodendrocytes. When PDGF levels are increased in the developing rat optic nerve oligodendrocytes cell death is decreased by up to 90% and the number of oligodendrocytes doubles in 4 days (Barres et al., 1992). There is an extremely fast rate of clearance of these apoptotic cells. It takes approximately one hour from when a cell dies to the time it is phagocytosed and degraded (Raff et al., 1993). Therefore normal oligodendrocyte death is a mechanism for adjusting the numbers of oligodendrocytes required to myelinate lengths of axon. In the adult optic nerve there are about 100,000 axons (Lam et al., 1982) myelinated by around 300,000 oligodendrocytes (Barres et al, 1992).

Survival of oligodendrocytes in the rat optic nerve is also dependent on the electrical activity of neighbouring axons. If the nerve axons are severed experimentally oligodendrocytes and astrocytes die (Barres and Raff, 1994). Axonal withdrawal also causes a downregulation of the major myelin proteins, MBP, PLP and MAG, at the transcription level in mature oligodendrocytes (Goto et al., 1990). Experimentally increasing PDGF levels in optic nerve can compensate. Hence axonal electrical activity is required to stimulate astrocytes to produce and release PDGF which keeps progenitor cells proliferating and inhibits cell death (Barres and Raff, 1993).

1.5 Some growth factors which regulate oligodendrocyte development

As has been briefly illustrated the development of oligodendrocytes from progenitor cells and the consequential production of myelin components are carefully regulated by a host of different factors. Many growth factors are known to be secreted by neurons and astrocytes *in vivo*, so both cell types are potential sources of molecules influencing oligodendrocyte differentiation and myelination. Described below are some growth factors which are involved in the regulation of oligodendrocyte development:-

1.5.1 PDGF

PDGF (Platelet derived growth factor) is a disulfide linked dimer of A and B chains with structure AA, BB or AB depending on its source (Collarini et al., 1991). Neurons have been shown to express A and or B chains (Yeh et al., 1991), and astrocytes to express A chains (Collarini et al., 1991), but it is unknown if neuron-derived PDGF contributes to oligodendrocyte development. PDGF binds to transmembrane receptors with extracellular ligand binding domains and intracellular tyrosine kinase domains (Reviewed by Collarini et al., 1991). Unoccupied receptors are monomeric but binding of PDGF induces dimerisation and activates tyrosine kinase activity. There are two types of PDGF receptor subunit; α binds both A and B chains but β binds only B chains. 0-2A progenitor cells express predominantly the α receptor (McKinnon et al., 1990). PDGF is both a survival factor and a mitogen for 0-2A progenitor cells. It stimulates 0-2A cell proliferation and prevents premature differentiation (McKinnon et al., 1993; McKinnon et al., 1990) which later proceeds by default when PDGF and other exogenous signals are removed (Temple and Raff, 1985). The concentration of PDGF required to promote oligodendrocyte precursor survival is 200 fold lower than that required to promote their proliferation and only PDGF-AA efficiently promotes survival (Barres et al., 1993). Possibly there is a high affinity PDGF receptor which signals survival but not proliferation.

Following differentiation PDGF receptors are downregulated in maturing oligodendrocytes (Pringle et al., 1992). In these postmitotic oligodendrocytes persisting PDGF mediates an increase in cytosolic Ca^{2+} which activates c-jun and c-fos expression. This Ca^{2+} mediated signalling pathway may cause process elongation in myelinating oligodendrocytes (Hart et al., 1989; Pfeiffer et al., 1993).

1.5.2 bFGF

bFGF (basic fibroblast growth factor) has been purified from many tissues which have been shown to be FGF sensitive. These include brain, pituitary, retina, corpus luteum, adrenal gland, kidney, placenta, macrophages, prostate and thymus (Gospodarowicz et al., 1986). bFGF is a single chain polypeptide composed of 146 amino acids which can also exist in a truncated form missing the first 15 amino acids. In the brain, pituitary and retina the complete form predominates (Esch et al., 1985). Acidic FGF is found only in the brain and retina and is 30-100 fold less potent than bFGF (Bohlen et al., 1985). Unlike other mitogens FGF is not actively degraded by cells possibly because the receptor is not tyrosine phosphorylated which

often assists entry of the receptor into the cell (Gospodarowicz et al., 1986).

FGF regulates expression of the oncogenes c-fos and c-myc which are also involved in cell proliferation (Muller et al., 1984). In cultured oligodendrocytes it stimulates cell migration, increases the rate of cell proliferation and prevents differentiation (Gospodarowicz et al., 1986). When purified 0-2A progenitor cells were cultured in the presence of very low doses of bFGF there was substantial differentiation in the presence of continued DNA synthesis, so it is possible to uncouple the inhibitory effects of bFGF on differentiation from its ability to promote DNA synthesis (Mayer et al., 1993). bFGF and PDGF are known to cooperate together to induce 0-2A cell proliferation and control differentiation (Boegler et al., 1990; McKinnon et al., 1993). The accumulation of PDGFα receptors on oligodendrocytic lineage cells is characteristic of bFGF signalling (McKinnon et al., 1990).

bFGF may participate in repair mechanisms of CNS myelin. It induces strong proliferation of 0-2A cells and slightly increases the number of MBP expressing oligodendrocytes. It also promotes the reconstruction of myelin-like membranes but in an uncompacted form (Fressinaud and Vallat, 1994). PDGF is as potent as bFGF in inducing 0-2A proliferation following injury, but PDGF is better at inducing the reappearance of more myelin like structures with better compaction (Fressinaud et al., 1996).

When purified 0-2A progenitor cells are cultured with both PDGF and bFGF and are therefore maintained in a proliferating state, the POU domain transcription factor SCIP/Tst-1 is expressed at high levels. This is downregulated when the growth factors are withdrawn and differentiation commences followed by the sequential expression of myelin specific proteins (Collarini et al., 1991). SCIP is expressed in proliferating Schwann cells of the PNS but is down regulated when they begin to express myelin specific genes (Monuki et al., 1989). It has been suggested that SCIP is a repressor of myelin gene expression in proliferating Schwann cells (Monuki et al., 1990) and this may also be the case in proliferating 0-2A cells. SCIP/Tst-1 is also expressed by other cell lineages in the CNS including neurons (Collarini et al., 1991) so may be mechanistically involved in the transition from proliferation to differentiation more generally.

1.5.3 CNTF

CNTF (Ciliary neurotrophic factor) is found in astrocytes in the CNS and promotes the survival and maturation of cultured oligodendrocytes. It protects oligodendrocytes from death induced by tumour necrosis factors (apoptosis) but not

against complement mediated lysis (necrosis) following injury. Thus CNTF plays a role in the survival of oligodendrocytes during development and its survival effect is restricted to the apoptotic mode of oligodendrocyte death during development or following exposure to environmental signals such as cytotoxic cytokines (Louis et al., 1993). CNTF protein and mRNA are found throughout the brain, mostly associated with astrocytes (Stoeckli et al., 1989) and the appearance of CNTF coincides with the time when oligodendrocytes are first generated from 0-2A progenitors. CNTF is able to induce a transient expression of GFAP in 0-2A progenitors from the optic nerve and initiate their differentiation into type 2 astrocytes but other signals are required to drive the process to completion (Hughes et al., 1988; Lillien et al., 1988).

1.5.4 IGF

IGF (Insulin-like growth factors). IGF-I and IGF-II are both potent inducers of oligodendrocyte development and support the survival of newly formed and mature oligodendrocytes (Barres et al., 1992; McMorris and Dubois-Dalcq, 1988). The addition of IGF-I to oligodendrocyte cultures increases the amount of CNP and CNP mRNA expressed per cell (Mozell and McMorris, 1991; van der Pal et al., 1988). It also increases the incorporation of sulfate into sulfolipids and increases myelin production. IGF-I and II and their mRNAs are detected in developing oligodendrocytes in fetal, neonatal and adult brain. Their pattern of peak expression corresponds to the major period of oligodendrocyte development (Adamo et al., 1989). The production of IGF-I, in some cases, by developing oligodendrocytes which also respond to it (autocrine action) support the theory that oligodendrocyte development may in part be autoregulatory (Shinar and McMorris, 1995). IGFs also regulate astrocyte and neuron development (Nielsen et al., 1991; Shemer et al., 1987).

1.5.5 NT3

NT3 (Neurotrophin 3) promotes the survival of purified 0-2A progenitor cells in vitro. It collaborates with PDGF to promote the clonal expansion of oligodendrocyte precursor cells in vitro and to drive the intrinsic clock that times their development (Barres et al., 1994). Cultured astrocytes and astrocytes purified from rat optic nerve secrete NT3 and NT3 mRNA has been detected in developing optic nerve (Elkabes et al., 1993). *In vivo* NT3 helps stimulate the proliferation of oligodendrocyte precursor cells and is thus required for normal oligodendrocyte development (Barres et al., 1994).

1.5.6 Neuregulins

The neuregulins are a family of soluble and transmembrane protein isoforms encoded by a single gene. They include glial growth factors (GGFs), neu differentiation factor (NDF), heregulins and acetylcholine receptor-inducing activity (ARIA) (Marchionni et al., 1993). The proteins mediate their effects by binding to receptor tyrosine kinases of the ErbB family, which are structurally related to the epidermal growth factor (EGF) receptor (Reviewed by Carraway and Burden, 1995). It is thought that neuregulins interact with a complex composed of erbB3 and erbB4, which heterodimerise in various combinations with each other, and erbB2 to effect subsequent signalling.

The neuregulins are expressed very early in CNS development well before oligodendrocyte precursors are thought to arise at around E16 in the rat forebrain (Levine and Goldman, 1988) and they persist at low levels into adulthood (Canoll et al., 1996). GGF has long been known as a mitogen for Schwann cells and astrocytes (Brockes et al., 1980) but the mitogenic response is greater in cells of the oligodendrocyte lineage. This correlates with the increased phosphorylation of neuregulin receptors in these cells (Canoll et al., 1996). Canoll et al. show that neuregulin receptors are expressed at various stages of the oligodendrocyte lineage including $04^+/01^-$ progenitor oligodendrocytes and mature $04^+/01^+$ oligodendrocytes. They also show that high levels of GGF reversibly inhibit differentiation and the lineage commitment of oligodendrocyte progenitors. In cultures of differentiated oligodendrocytes it causes the loss of 01 and MBP expression (Canoll et al., 1996). Therefore a role of neuregulins is to promote oligodendrocyte proliferation whilst preventing premature differentiation.

Neurons are an important source of oligodendroglial mitogens (Barres and Raff, 1994), releasing soluble factors such as PDGF (Dutly and Schwab, 1991) and bFGF (Hardy and Reynolds, 1993) and stimulating proliferation via contact-dependent factors (Chen and DeVries, 1989). Cortical neurons express high levels of GGF during development (Chen et al., 1994). GGF and other neuregulins may also function as axolemma-associated mitogens as is the case for Schwann cells. Norlund et al. described the partial purification of a 50 kD protein from brain membrane preparations which is mitogenic for both Schwann cells and oligodendrocytes and may correspond to GGF (Norlund et al., 1992). Axolemma -associated factors inhibit the differentiation of oligodendrocytes and promote their proliferation (Zajicek and Compston, 1994). During CNS development the neuregulin receptors on oligodendrocytes may be down regulated or other factors

released by astrocytes may overcome this inhibition, thus promoting differentiation (Canoll et al., 1996).

Based on in situ hybridisation experiments, neurons and cells of the sub-ventricular zone (SVZ) appear to be major sources of neuregulins in the developing and adult CNS (Corfas et al., 1995). In addition astrocytes also synthesise these proteins (Pinkas-Kramarski et al., 1994). The exact identity of the cells expressing neuregulins in the SVZ is unknown but this is a region containing mostly glial progenitor cells in the early postnatal period. This suggests that neurons and glia are important sources of neuregulins during CNS development (Canoll et al., 1996).

1.6 Oligodendrocyte progenitors in the adult nervous system.

During development, as previously discussed, oligodendrocytes develop from oligodendrocyte progenitor cells but in the adult nervous system, where the turnover of oligodendrocytes and astrocytes is extremely slow, do they still arise from the same progenitor cells which are just maintained in situ until required? Progenitor cells which replace the myelinating population as it slowly turns over have been isolated from adult optic nerve and spinal cord and have been analysed *in vivo* (Wolswijk and Noble, 1989; Engel and Wolswijk, 1996). These adult progenitor cells have different characteristics in culture from their perinatal counterparts. Both the adult and perinatal 0-2A progenitor cells are labelled with the A2B5 antibody but adult cells also label with 04 which recognises only a subpopulation of perinatal progenitors (Wren et al., 1992). Adult progenitors are vimentin negative, proliferate and differentiate more slowly than perinatal cells and are susceptible to the lytic effects of complement, unlike perinatal 0-2A cells (Wolswijk and Noble, 1989; Wren et al, 1992; Engel and Wolswijk, 1996).

By one month after birth oligodendrocyte progenitor cells have disappeared from optic nerve and are not detectable in cultures from adult optic nerves (Wolswijk and Noble, 1989). Hence this progenitor cell population has a limited lifespan. They are replaced by the adult progenitor cells which are fist detected at P7 and can still be identified in optic nerves from one year old rats (Wolswijk et al., 1990). The adult progenitor cell arises from a subpopulation of the perinatal cells as demonstrated in culture when, after an initial proliferation period driven by type 1 astrocytes, 0-2A cells isolated from the optic nerve will generate an oligodendrocyte precursor cell which differs from the 0-2A cell (Wren et al., 1992). This new cell demonstrates characteristics of the adult progenitor cell. The generation of oligodendrocytes in vitro from these adult progenitor cells occurs by asymmetric division and differentiation so that there is constant renewal of these precursors

(Wren et al., 1992). Hence they behave as a type of stem cell. Adult progenitor cells also express other properties of stem cells such as their long cell cycle times (Wolswijk et al., 1990; Wolswijk and Noble, 1989; Wolswijk et al., 1991) and the ability to generate differentiated progeny as well as self renewal throughout life (Wren et al., 1992).

It was previously believed that perinatal progenitor cells divided symmetrically in vitro eventually resulting in self extinguishment (Raff et al., 1988; Temple and Raff, 1986). This is indeed true when 0-2A progenitor cells derived from perinatal rat optic nerve are cultured in the presence of astrocytes which secrete factors promoting oligodendrocyte differentiation. However, recently it was noted that in cultures derived from E15 rat brain, oligodendrocytes were generated by asymmetric division and differentiation of progenitor cells and that the extent of oligodendrocyte generation but not the probability was modulated by extrinsic factors such as thyroid hormone (T3) (Ibarrola et al., 1996). A new hypothesis has therefore been formed suggesting that the initial generation of oligodendrocytes is generally by asymmetric division and differentiation of progenitor cells and that this is a cell intrinsic property. The extent of differentiation can be modulated by cell extrinsic factors e.g. T3, CNTF and NT-3. An intrinsic clock measures the mitotic lifespan of the progenitor cells and when the time ends, if environmental signals permit, most cells will become oligodendrocytes. A second pathway may also exist to generate adult progenitor cells (Ibarrola et al., 1996).

The adult 0-2A progenitor cell described above is detected in normal nervous tissue, but is it also able to replace damaged oligodendrocytes following injury? This seems an unlikely possibility considering its susceptibility to the lytic effects of complement which are activated as a consequence of injury and induction of the immune system. A progenitor cell was identified in lesioned areas of adult cat optic nerve which differed from adult 0-2A cells but had similarities to the perinatal 0-2A progenitor cell (Carroll and Jennings, 1994). It had fast cell cycle times of approximately 18 hours, was vimentin positive, highly motile becoming closely apposed to demyelinated axons, was resistant to the lytic effects of complement which were highly active in the inflamed region of injury and it differentiated into oligodendrocytes. This cell possibly arose as a result of division by a putative resting progenitor cell in the normal optic nerve surrounding the lesion. Such a readily recruitable and motile progenitor cell which is unaffected by the immune system has many important implications in myelin repair mechanisms following injury or demyelination as a result of viral infection, when a rapid response is also required. Therefore normal adult 0-2A progenitor cells are apparently responsible

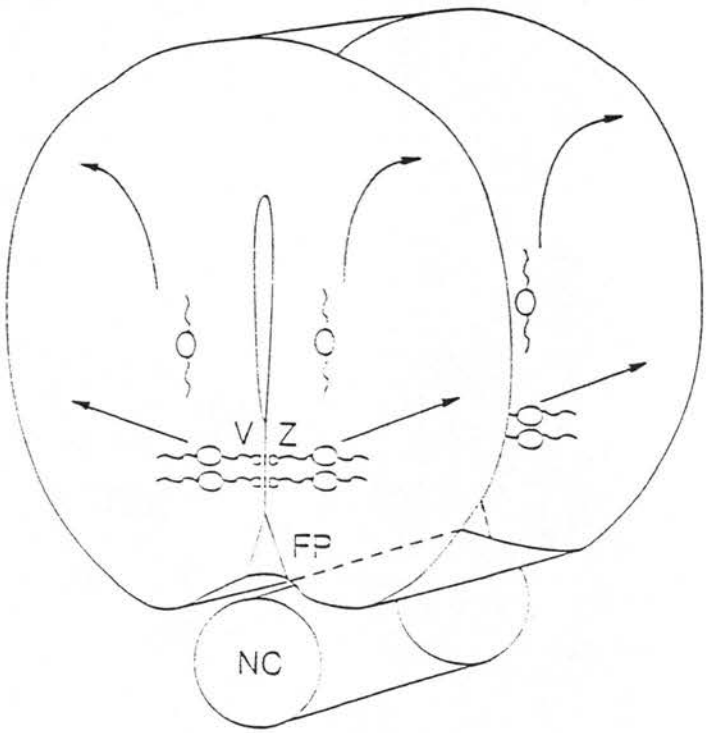
for the general maintenance of the nervous system, replacing cells as and when required due to general cell turnover. A second faster responding cell type is recruited in emergency situations when damage to the nervous system must be minimised. This hypothesis is supported by the fact that when $04^{+}GC^{-}$ adult progenitor cells are treated with PDGF:and bFGF, which are both upregulated following CNS injury (Lotan and Schwarz, 1992), a small population transiently reverts to a more mitogenically active and migratory phenotype with similarities to the perinatal 0-2A progenitor cell (Wolswijk and Noble, 1992). Normal adult progenitor cells are found evenly distributed throughout the optic nerve and represent up to 5% of the normal macroglial population (Fulton et al., 1992). Signals from a lesion may cause some of these to revert to a phenotype similar to perinatal 0-2A progenitor cells and respond to the injury by differentiating into oligodendrocytes which subsequently repair the damage by remyelination.

1.7 Embryonic origin of glial progenitors.

Oligodendrocyte precursors, the 0-2A progenitor cells are thought to originate from the neuroectodermal cells of subventricular zones. The earliest identified precursor cells are proliferative, monopolar cells expressing vimentin which differentiate into proliferative, migratory, bipolar 0-2A precursor cells expressing GD3 (Hardy and Reynolds, 1991). GD3 is a major glycolipid component of the immature vertebrate CNS which is downregulated during early development. Its expression characterises immature dividing neuroectodermal cells such as the glial precursors (Goldman et al., 1984; Goldman and Reynolds, 1996). Vimentin is a marker for undifferentiated neuroepithelial cells following the closure of the neural tube (Bignami et al., 1982). The $GD3^{+}$ 0-2A precursors are located in the subependymal layers of the 4th ventricle and migrate from there to their final positions in the brain. As they reach their final destinations they differentiate via a pro-oligodendrocyte (04^{+}, GC^{-}) into GC expressing oligodendrocytes (04^{+}, GC^{+}) which then synthesise myelin. Consistent with overall brain development oligodendrocytes develop in a posterior to anterior gradient in the brain (Hardy and Reynolds, 1991; Reynolds and Wilkin, 1988).

Studies on spinal cord, one of the first parts of the CNS to be myelinated, provide evidence to suggest that oligodendrocytes are generated from the ventral half of the neural tube but it is still controversial whether they also arise from the dorsal half. It has also been suggested that oligodendrocytes in the spinal cord can develop from dedifferentiated radial glia (Choi et al., 1983; Hirano and Goldman, 1988). Expression of the PDGFα receptor (PDGFαR) has been used to identify cells of the

Figure 1.4 Schematic diagram of the developing vertebrate spinal cord demonstrating the embryonic origin of oligodendrocytes in the ventral ventricular zone (VZ) and their subsequent migration dorsally and radially (arrows). FP=floor plate and NC=notochord. (Figure from Miller, 1996)


V
Z
FP
NC

oligodendrocyte lineage (Pringle et al., 1992). Investigations show that PDGFαR+ cells originate at a defined point in the ventricular zone and subsequently migrate away from there into the surrounding tissue (Pringle and Richardson, 1993). Hall et al. recently demonstrated that all PDGFαR+ cells in the embryonic rat spinal cord were oligodendrocyte precursors (Hall et al., 1996). Other studies suggest that oligodendrocyte precursors arise in the ventral ventricular zone of the embryonic neural tube from which they spread first over the ventral then over the dorsal halves of developing white matter (Cameron-Curry and Le Douarin, 1995). In the developing chick spinal cord oligodendrocyte precursors are specifically labelled with 04 (Ono et al., 1995). This immunoreactivity appears very early and its expression is retained throughout cellular maturation. The earliest 04+ cells are located in the ventral ventricular zone and subsequently migrate into the dorsal region. In the avian spinal cord Cameron-Curry (Cameron-Curry and Le Douarin, 1995) claims that cells from dorsally located precursors migrate to dorsal and lateral funiculi and to more ventrally located regions. However when dorsal and ventral regions of E4 chick spinal cord were cultivated separately significant numbers of oligodendrocytes failed to develop in dorsal spinal cord cultures but E7 dorsal spinal cord was found to produce large numbers of oligodendrocytes. Hence there is a limited period when oligodendrocyte potentiality is restricted to the ventral region. When E4 dorsal spinal cord microexplants were co-cultivated with notochord segments or with floor plate tissue, oligodendrocytes were found but when explants were cultivated alone no oligodendrocytes were detected (Trousse et al., 1995). It therefore seems that there are cells present in the dorsal spinal cord which can be induced to differentiate into the oligodendrocyte phenotype by ventrally derived signals from notochord and floor plate.

Neural crest cells of the dorsal neural tube are multipotent and following detachment they migrate to diverse locations in the developing embryo where they give rise to a number of different cell types including neurons, glia, secretory cells of the peripheral neuroendocrine system, melanocytes, chondrocytes and smooth myocytes. Hence a homogeneous population of non differentiated cells are channelled into different developmental pathways (Stemple and Anderson, 1992). *In vitro* studies have demonstrated that some multipotent cortical cells are able to self renew, a property of stem cells (Davis and Temple, 1994), and there is some evidence for the existence of such cells *in vivo* (Reid et al., 1995). It is therefore possible that neurons and glia in the CNS may arise from multipotent founder cells via restricted progenitor cells.

The development of neurons and oligodendrocytes from a common precursor

has been demonstrated in vitro (Williams et al., 1991). In cultures of dissociated embryonic rat cerebral cortex where a small number of cells are infected with a recombinant retrovirus carrying the lacZ gene and which can be detected by anti-β-galactosidase serum and identified by monoclonal antibodies, most precursors generated either neurons or glia. Some neuronal clones however also included oligodendrocytes and the proportion of mixed clones increased from E12, a time before neurogenesis begins in the cortex, until E16, the peak time of neurogenesis (Miller, 1986), when 18% of neuronal clones also contained oligodendrocytes. Whether the fate of progenitor cells *in vivo* is specified intrinsically or through environmental factors is unknown. However Qian and colleagues have recently demonstrated that bFGF seems to influence the fate of embryonic cortical stem cells *in vitro* (Qian et al, 1997). They demonstrate that when cells from the cortical ventricular zone are cultured in the presence of low levels of bFGF the predominant cell fate was neuronal. At higher concentrations of bFGF both neurons and glia developed. It was suggested that the neuronal lineage was the default pathway of cell development but when bFGF levels reached a threshold level cells were produced with the bipotential to generate oligodendrocytes or astrocytes. Formation of oligodendrocytes was then the default pathway of development with additional signals required for astrocyte formation. This finding is consistent with recent work demonstrating how bone morphogenic proteins (BMPs) cause cells which have been generated from the sub ventricular zone (SVZ), a region arising later from the ventricular zone, to become astrocytes (Gross et al., 1996). This is associated with almost complete suppression of neuronal and oligodendrocyte lineage elaboration. The SVZ progenitor cells were also shown to express BMP-specific type I and type II receptor subunits and selective BMP ligands indicating either paracrine and/or autocrine developmental signalling pathways. Therefore the fate choice of multipotent cortical stem cells is at least partly directed by environmental signals.

1.8 A differentiated glial subtractive library

How can we gain a better understanding of a) the regulation of oligodendrocyte development from progenitor cells in both the developing nervous system and in adults, b) how oligodendrocytes get to their correct locations to do their job, c) how other cells such as astrocytes and neurons are involved and what exactly they do, and d) whether axons signal to induce myelination in development and in response to injury and what these signals are? Gaining an understanding in just some of these areas may also eventually lead to therapies for many disorders and diseases of the nervous system such as multiple sclerosis in humans. Knowledge

and understanding at the genetic level could also contribute to the correction of inherited disorders such as the multiple forms of Pelizaeus-Merzbacher disease (PMD). The appearance of novel proteins which may also become upregulated at particular times of development or in particular situations may implicate their involvement in particular events. Perhaps by identifying all the pieces involved, the jigsaw can be constructed.

The total number of genes expressed in the mammalian brain has been estimated at approximately 30,000 with at least half of them being present exclusively in brain (Bantle, 1987; Chikaraishi, 1979; Milner, 1983). Systematic analysis therefore of individual novel brain specific mRNAs potentially offers important insights into neural function and the composition of the nervous system. Well over 100 brain specific proteins have already been identified biochemically through protein purification, by functional assays, or by the isolation and characterisation of mRNAs from brain cDNA libraries (Sutcliffe, 1988). Many of these early-identified brain specific proteins are relatively abundant such as the neurofilament proteins and the major constituents of myelin but many more, including as yet unidentified proteins, are extremely rare yet may well have important roles to play in myelin structure, cell-cell interactions, the regulation of development or in cellular plasticity during development or repair of the nervous system.

Several powerful methods have been developed to allow the isolation of such rare and specifically expressed mRNAs. The main techniques are differential screening of cDNA libraries, differential display and subtractive hybridisation. All these techniques compare two mRNA populations e.g. from different time points in development and enable the identification of mRNAs present in one but not the other. Typically such isolated clones represent genes which alter their expression during development, or as a consequence of disease, or due to an induction signal.

In differential screening probes are made from the mRNAs of two populations being compared. They are then used to screen a cDNA library. Clones which hybridise to one probe but not to the other are isolated and analysed further. This technique is able to detect relatively abundant transcripts representing approximately 0.1% of an mRNA population (Ausubel et al., 1992) In differential display partially random primers are used to amplify a subset of mRNAs expressed in a given population. These are separated on an acrylamide gel and the bands between different samples compared. This technique generally targets medium abundant mRNAs but can yield many false positives (Ausubel et al., 1992) The third main technique is subtractive hybridisation. A cDNA library representing one

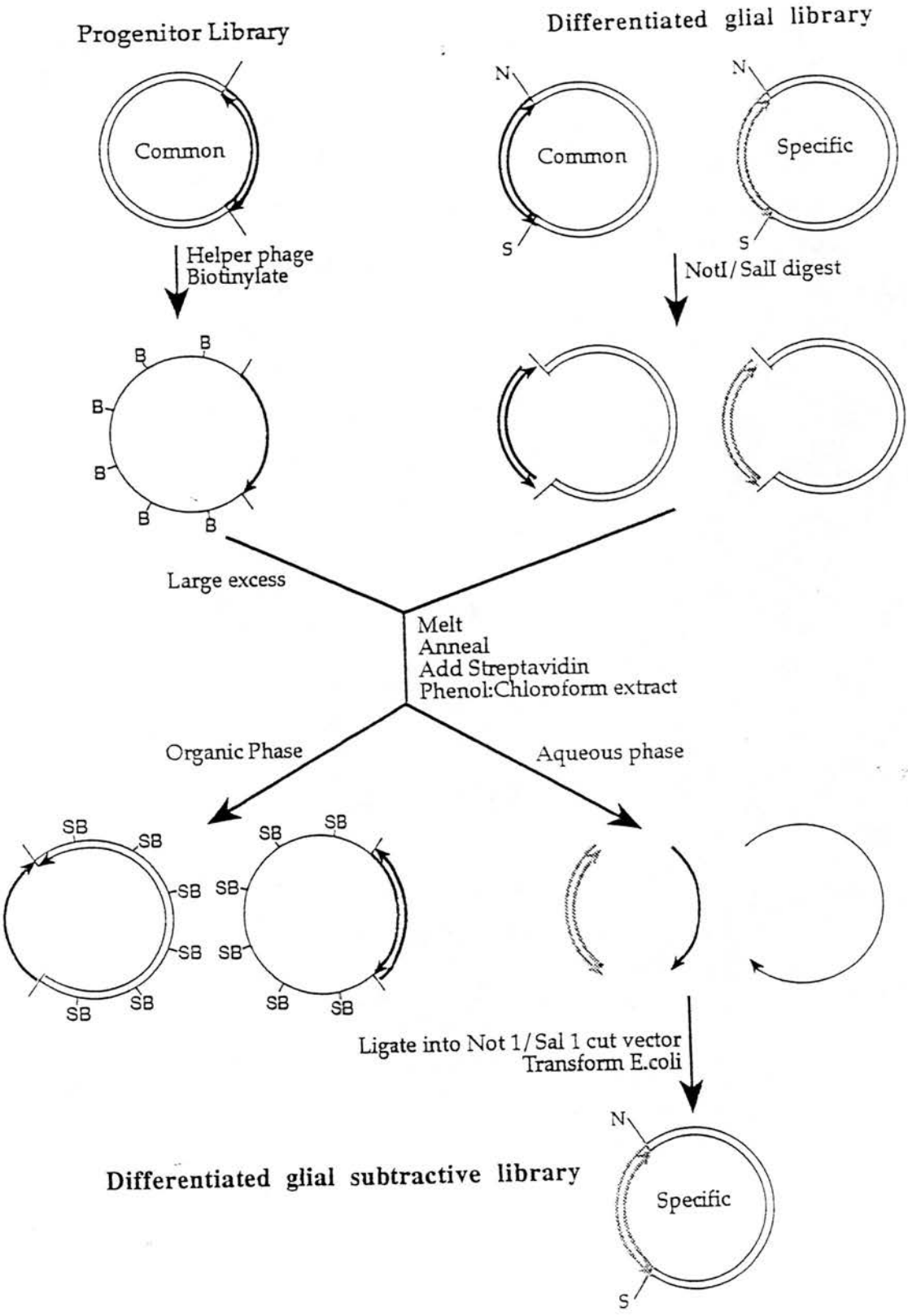
population is used to remove all sequences which are also present in a second library representing another population. The resultant subtracted cDNA library contains cDNA clones which are present exclusively in the second population. Of all three techniques subtractive cloning is the most sensitive, due to its enriching steps, enabling detection of rare transcripts forming less than 0.001% of an mRNA population. It is also the best method for isolating as complete a set of differentially expressed genes as possible (Ausubel et al. 1992).

A combination of differential screening and subtractive hybridisation has also proved useful whereby a cDNA library is hybridised with probes which have been enriched for by subtractive hybridisation (Baba, 1994). A number of interesting neural proteins identified by subtractive screening include RC3, a calmodulin binding, protein kinase C substrate which is enriched in the dendrites of forebrain neurons (Watson, 1990), the retinal degeneration slow gene product (Travis, 1989), PMP22, the locus of the trembler mouse mutant and the human genetic disorder, Charcot-Marie-Tooth disease(Patel, 1992; Suter, 1992) and MOBP (Schaeren-Wiemers et al., 1995).

In the present study clones were isolated from a cDNA library which was the product of subtractive hybridisation between a library encoding clones expressed by glial 0-2A progenitor cells and a second library encoding clones expressed by glial cells which had been allowed to differentiate in culture for three days. Isolated 0-2A progenitor cells from mixed glial cultures established from neonatal rat brains, were used to make the first cDNA library. The second cDNA library was prepared from cells which had differentiated from 0-2A cells in culture for three days. The culture medium contained low amounts of FCS so the majority of differentiated glia were oligodendrocytes but some astrocytes were also present. Hence this second library represented clones expressed by differentiated glia and not just by oligodendrocytes.

Figure 1.5 shows schematically how the subtractive library was constructed according to the method of Herfort and Garber, 1991. The subtraction was done by P.J. Brophy using cDNA libraries prepared by C.S. Gillespie. Biotinylated single stranded cDNA was produced from the progenitor library. The differentiated glial library was digested with the restriction enzymes NotI and SalI to release the insert sequences from the pSPORT1 plasmid vector. These cDNA fragments were heated to melt the double strands of DNA into single strands which were then mixed with the biotinylated single stranded cDNAs of the progenitor library. On cooling complementary sequences hybridised to each other, so vector sequences common to both libraries rehybridised, as did common insert sequences. Insert sequences which were unique to differentiated glia rehybridised with one another. Some unpaired

Figure 1.5 Schematic diagram demonstrating how the differentiated glial subtractive library was constructed from two cDNA libraries. A cDNA library encoding clones expressed by 0-2A progenitor cells was subtracted from a cDNA library encoding clones expressed by differentiated glial cells. The resulting subtractive library encodes cDNAs expressed specifically by differentiated glia. For details see the main text.


Progenitor Library
Differentiated glial library
Common
Common
Specific
N
S
Helper phage
Biotinylate
NotI/SalI digest
B
Large excess
Melt
Anneal
Add Streptavidin
Phenol:Chloroform extract
Organic Phase
Aqueous phase
SB
Ligate into Not 1/Sal 1 cut vector
Transform E.coli
Differentiated glial subtractive library
Specific

vector and insert fragments were also present. The addition of streptavidin, which binds to biotin, allowed the removal of all biotin containing cDNAs and therefore those which were common to both libraries, in the organic phase of a phenol chloroform extraction. What should remain in the aqueous phase are double stranded cDNAs encoding sequences specifically expressed by differentiated glia. These were ligated into the vector pSPORT1 and electroporated into E. coli DH12S to form the differentiated glial subtractive library which has a complexity of approximately 300,000.

1.9 The proposed study

This differentiated glial subtractive library is now a source of cDNA clones which are expressed following differentiation into glial cells. Since their expression must therefore be switched on sometime during the process of differentiation it is likely that the products of these cDNAs are involved somehow in glial development. Some of the clones in the library will be expressed exclusively by oligodendrocytes and therefore may be intrinsically involved in oligodendrocyte development, perhaps as a structural component of the myelin sheath. Other clones will also be expressed by different cell types such as astrocytes and neurons and so may be part of the physical interactions between these cells or as components of signalling mechanisms between cells. There will also be clones which are expressed in addition by other cell types outside of the nervous system. These may represent as yet unrecognised components necessary for more general mechanisms of differentiation exhibited throughout development.

In this study individual clones were isolated from the subtractive library with the intention of identifying novel proteins expressed by differentiated glial cells and with a view to eventually elucidating their role in the developing nervous system. From this differentiated glial subtractive library five novel clones were isolated which are expressed specifically by the nervous system. One was chosen for detailed investigation.

CHAPTER 2. MATERIALS AND METHODS

2.1 NORTHERN BLOTTING

2.1.1 Extraction of total RNA from rat tissues

DEPC-treated MilliQ dH_2O was prepared by the addition of Diethyl pyrocarbonate (DEPC, Sigma) (500 µl) to MilliQ dH_2O (500 ml). After mixing, the solution was left to stand at RT overnight and then autoclaved.

P10 Wistar rats were sacrificed. Various tissues were rapidly dissected from the animal using sterile instruments, loosely wrapped in aluminium foil and frozen in liquid nitrogen. The dissected tissues were stored at -70°C until required.

Tissue samples were homogenised with RNAzol B (Biogenesis, Bournemouth, England) (approximately 2 ml per 100 mg of tissue) using a few strokes in a glass-Teflon homogeniser. The resulting homogenate was divided into aliquots (1 ml) in 1.5 ml Eppendorf tubes on ice. Chloroform (100 µl) was added to each tube which was then vortexed to mix and left on ice for 5 min. The tubes were centrifuged at 13,000 g for 15 min at 4°C in a benchtop centrifuge (Biofuge 13, Heraeus Instruments). The top aqueous phase containing soluble RNA was then carefully removed to a new Eppendorf tube. An equal volume of phenol/chloroform (1:1) was added, the tubes vortexed and then centrifuged at 13,000 g for 5 min at 4°C. Again the top aqueous layer was carefully removed to a clean Eppendorf tube and the phenol/chloroform extraction repeated. This was done until there was no precipitate of DNA/protein visible at the interphase of the aqueous and organic phases (2-3 times should be sufficient). To the isolated upper aqueous phase an equal volume of isopropanol was then added. The tubes were vortexed and left on ice for 15 min to precipitate the RNA. Centrifugation at 13,000 g for 45 min at 4°C pelleted the RNA. The supernatant was carefully removed and the pellet washed in 70% ethanol (500 µl). After centrifugation at 13,000 g for 5 min at RT and removal of the supernatant the pellet was allowed to briefly air dry before being resuspended in DEPC treated MilliQ dH_2O.

2.1.2 Spectrophotometric determination of RNA purity and yield

Spectrophotometric measurements (LKB-Ultrospec III, Pharmacia Biotech) of the RNA samples were used to calculate purity and concentration. Blank readings to set the references were done on 1 ml of MilliQ dH_2O in a quartz cuvette. Sample readings were done on aliquots of sample RNA (2 µl) diluted 500 times with MilliQ dH_2O to a final volume of 1ml in the cuvette. The optical density (O.D.) of the samples were measured at 260 nm to estimate nucleic acid content and at 280 nm to estimate protein content. Purity of the RNA sample is determined by a ratio of protein to nucleic acid content. A figure of between 1.7 and 2.0 is acceptable.

2.1.3 Filter preparation for Northern Blotting

For 200 µl of RNA denaturation buffer, which was freshly prepared as required:-

20 µl 10x MOPS buffer (0.2 M MOPS, 50 mM sodium acetate, 10 mM EDTA; pH 7)
100 µl formamide
40 µl formaldehyde
20 µl ethidium bromide (0.5 mg/ml, w/v)
20 µl DEPC dH_2O

RNA denaturation buffer (10 µl) was added to an aliquot of RNA (10 µg) in a 0.5 ml Eppendorf tube. The tubes were incubated at 65°C for 20-30 min to denature the RNA and then plunged onto ice. After a brief pulse centrifuge, filtered loading buffer (30% Ficol Type 400 (Pharmacia), 0.25% bromophenol blue) (1 µl) was added. Samples were run on a 0.8% formaldehyde agarose gel in 1x MOPS buffer at 50 V for 3-4 h. Size standards (0.24-9.5 kb RNA ladder, GIBCO BRL) were also run on the gel.

For a 0.8% gel:- 4 ml 10x MOPS buffer
1.2 ml formaldehyde
34.8 ml 1% agarose (Seakem, FMC Bioproduct) (w/v) in MilliQ dH_2O

The RNA was then transferred onto Hybond-N nylon membrane (Amersham) under vacuum at 40 mBar for 90 min whilst submerged in 20x SSC (3 M NaCl, 0.3 M trisodium citrate, pH 7). After transfer and before drying, the filter was briefly viewed with a U.V. light source and the size standards noted with pencil for reference later. The gels could then be discarded. Once the filters were dry they were baked in an oven at 80°C for 1.5-2 h and stored at RT until required.

2.1.4 Probe preparation for Northern Blotting

Plasmid DNA was radioactively labelled with [α-^{32}P] dCTP (Amersham) using the Random Priming Kit (GIBCO, BRL). Plasmid DNA (25 ng) in a volume of 10 µl was boiled for 5 min, then plunged onto ice. Then added were ATP, GTP and TTP (2 µl of each), Random primers buffer (15 µl), dH_2O (13 µl), [α-^{32}P] dCTP (5 µl, equal to 50 µCi) and Klenow fragment (1 µl). The tube was mixed gently and incubated at 25°C for 1 h before the addition of stop solution (5 µl). Sheared salmon sperm DNA (100µl of a 10mg/ml stock) (Stratagene) was then added. At this stage the probes could be stored overnight at -70°C or used immediately.

2.1.5 Northern Blotting-Hybridisation

The prepared filters were trimmed to a minimum size, rinsed very briefly in dH_2O to remove excess salts and then placed in individual 50 ml universal tubes with QuikHyb Hybridisation Solution (3 ml) (Stratagene). The filters were pre-hybridised at 65°C for 30 min on a rotary oven.

The radioactively labelled cDNA probe and salmon sperm DNA were boiled for 5 min (minimum) and then rapidly added to the pre-hybridising Quikhyb solution. The filters were left to hybridise with the probes for 1.5 h at 65°C in the rotary oven.

Filters were then washed at low stringency twice for 10 min in 100 ml of 2xSSC/0.1%SDS with agitation at RT, then at high stringency twice for 30 min in 40 ml of 0.2xSSC/0.1%SDS at 65°C in the rotary oven.

Filters were wrapped in Saran-Wrap while still damp and exposed to photographic film (Agfa Curix RPI) in a cassette containing an intensifying screen. Films were developed using chemicals from Kodak: Kodak LX24 X-ray developer diluted 1:18 with tap water and Kodak Industrex manual fixer diluted 1:4 with tap water.

2.2 OLIGODENDROCYTE CULTURES

1-2 day old Wistar rats were decapitated and the heads swabbed with 70% ethanol. The cerebral hemispheres were then quickly removed and placed in a Petri dish containing approximately 1.5 ml per brain of cold 1x Hank's balanced salt solution without Ca^{2+} or Mg^{2+} (HBSS-Ca, Mg). The tissue was dissociated as much as possible by chopping with scissors and was then forced through a 140 µm sterile nylon mesh attached to a cut off 10 ml syringe, then through another of 35 µm. (Nybolt, Plastock Assoc., Birkenhead, UK) Each mesh was washed through with 1x HBSS to minimise cell loss.

Cells were harvested by centrifugation at 1000 g for 3 min at RT. The supernatant was carefully removed and discarded. The pellet was gently resuspended in Dulbecco's modified Eagle's medium without Glutamine (DMEM) containing 10% foetal calf serum (FCS) (Flow, UK), penicillin (100 IU/ml) and streptomycin (100 µg/ml) (both from ICN). Approximately 10 brains were used to seed 8x 75cm^2 flasks (Costar) which had been coated with 0.1 mg/ml poly-D-lysine hydrobromide (30-70,000 M_r, Sigma) for 30 min. Each flask contained a final culture volume of 11 ml. These primary cultures were maintained in a 37°C incubator with a water saturated atmosphere and 5% CO_2.

culture volume of 11 ml. These primary cultures were maintained in a 37°C incubator with a water saturated atmosphere and 5% CO_2.

After 3-4 days the medium was replaced with DMEM containing 10% FCS, penicillin (100 IU/ml), streptomycin (100 µg/ml) and glutamine (2 mM) (ICN).

After 6-10 days in culture a clearly defined bilayer of cells formed with phase-dark rounded O-2A progenitors in the top layer. The flasks were sealed and pre-shaken at 180 rpm for 2 h at 37°C in an orbital incubator to remove loosely attached macrophages. The medium was then replaced with fresh DMEM containing 10% FCS, penicillin (100 IU/ml), streptomycin (100 µg/ml) and glutamine (2 mM) and the cultures allowed to equilibriate for at least 2 h in the incubator at 37°C with a water saturated atmosphere and 5% CO_2 . Then the flasks were resealed and shaken at 180 rpm for 18-24 h in the 37°C orbital incubator.

The monoclonal antibody A2B5 (200 µl) was then added to each flask which was then inverted so that the roof of the flask became the adhesion surface. The flasks were placed in the incubator for 5-20 min or until the flat cells and remaining macrophages had adhered, leaving the small round O-2A progenitor cells coated with the A2B5 monoclonal antibody in suspension. These cells could then be removed to a 50 ml universal tube and harvested by centrifugation at 1000 g for 3 min at RT. The pellet was gently resuspended in Defined Medium (+bFGF/-FCS) (DMEM containing transferrin (50 µg/ml), triiodothyronine (30 nM), NaSelenite (20 nM), D-biotin (10 ng/ml), progesterone (20 nM), putrescine (100 µM), insulin (5 µg/ml), glutamine (4 mM), penicillin (100 IU/ml), streptomycin (100 µg/ml) and bFGF (2 ng/ml). All supplements were from Sigma except penicillin-streptomycin and glutamine which were from ICN and bFGF which was from British Biotechnology, Oxford, UK).

The resuspended cells from 8x 75 cm^2 flasks were seeded into 6x 25 cm^2 flasks each with 4 ml of medium or 20-30 µl were seeded per 13 mm glass coverslip (No1 thickness, BDH, UK). Up to 3 coverslips were maintained in 2 ml of medium in a 35 mm Petri dish.

The seeded progenitor cells were maintained in Defined Medium (+bFGF/-FCS) in the incubator (37°C, 5% CO_2, water saturated atmosphere) for 4 days, replacing half the medium every 24 h. The medium was then replaced with Defined Medium (-bFGF/+FCS) (DMEM containing the same supplements as above except without bFGF but with 1% FCS). The cells were allowed to differentiate in the presence of FCS for 4 days at 37°C with a water saturated atmosphere and 5% CO_2.

2.3 IN SITU HYBRIDISATION (ISH)

2.3.1 Plasmid DNA preparation

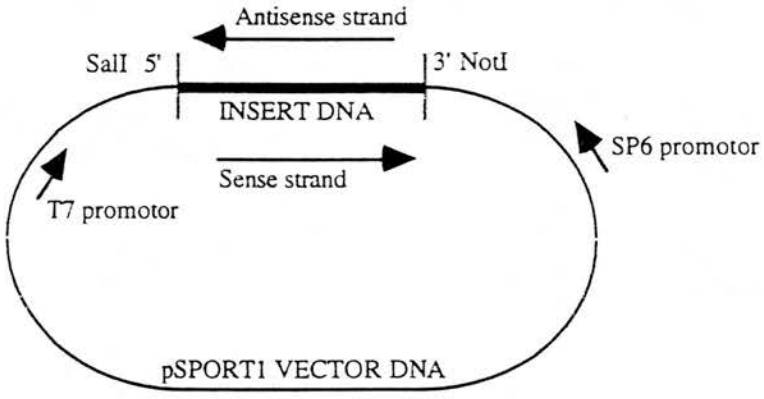
An aliquot (1 µl) of a frozen glycerol stock of XL-1 Blue E.coli cells containing the plasmid DNA of interest was added to 10 ml of L-broth (Luria Broth base, Sigma) containing ampicillin (100 µg/ml) in a 50 ml universal tube. The culture was incubated at 37°C in an orbital incubator (220 rpm) overnight.

The culture was centrifuged in a 30 ml glass corex tube at 9500 rpm for 10 min at 4°C (Sorvall RC 5B, rotor SS-34). The supernatant was discarded and the pellet resuspended in 1 ml of filter sterilised solution P1 (50 mM glucose, 10 mM EDTA pH 8, 25 mM Tris-HCl pH 8). Then 1 ml of freshly prepared solution P2 (1% SDS, 0.2 M NaOH) was added. The tube was mixed gently and left at RT for 5 min. Then 1ml of solution P3 (29.4 g potassium acetate, 11.5 ml glacial acetic acid, dH_2O to 100 ml, 3 M with respect to potassium and 5 M with respect to acetate) was added and the tube mixed by inversion. It was then centrifuged at 9500 rpm for 15 min at 4°C (Sorvall RC 5B, rotor SS-34). The supernatant was dispensed into a clean 30 ml corex tube and 0.8x the volume of isopropanol (2.4 ml) was added. After leaving on ice for 10 min, the tube was centrifuged at 9500 rpm for 20 min at 4°C. The supernatant was discarded and the pellet resuspended in 0.35 ml of TE (10 mM Tris/HCl pH 7.4, 1 mM EDTA pH 8) buffer. The solution was transferred to a 1.5 ml Eppendorf tube and 0.17 ml of LiCl (15 M) was added. After mixing it was left to stand at RT for 10 min. Centrifugation at 13,000 g for 15 min at RT pelleted the RNA. The supernatant was dispensed into a clean Eppendorf tube and 0.5 ml of phenol/chloroform (1:1) was added. After vortexing to mix and centrifugation at 13,000 g for 5 min at RT, the upper aqueous layer was removed to a clean Eppendorf tube where 0.1x the volume of 3 M Na Acetate, pH 5.5 and 2x the volume of 100% ethanol were added. After 30 min at -70°C, the tube was centrifuged at 13,000 g for 40 min at 4°C. The supernatant was discarded and the pellet washed with 70% ethanol (500 µl). It was then centrifuged at 13,000 g for 5 min at RT. After discarding the supernatant the pellet was briefly air-dried before resuspending it in TE buffer (40 µl).

A restriction digest was done on the purified plasmid DNA to confirm the presence of the correct-sized insert of interest and also to estimate plasmid concentration. An aliquot (1-2 µl) of the plasmid DNA was mixed with 10x restriction buffer H (Sure/Cut BufferH, Boehringer Mannheim) and the restriction enzymes NotI (5 U) (New England Biolabs) and SalI (5 U) (Advanced Biotechnologies) in a total volume of 10 µl. The digest was incubated at 37°C for 1-2 h and then run out on a 1% agarose electrophoresis gel in 1x TAE buffer (0.04 M

Tris-Acatate, 0.00 1M EDTA) at 50 mA to separate the vector (pSPORT1) which runs at 4 kb and insert DNA bands. A DNA mass/size ladder (Low or High DNA mass ladders from GIBCO) were used to estimate the size of the insert DNA band and the plasmid concentration. The plasmid DNA was stored at -20°C. Figure 2.1 shows a diagram of the pSPORT1 plasmid and its cloning sites.

2.3.2 Linearisation of plasmid DNA

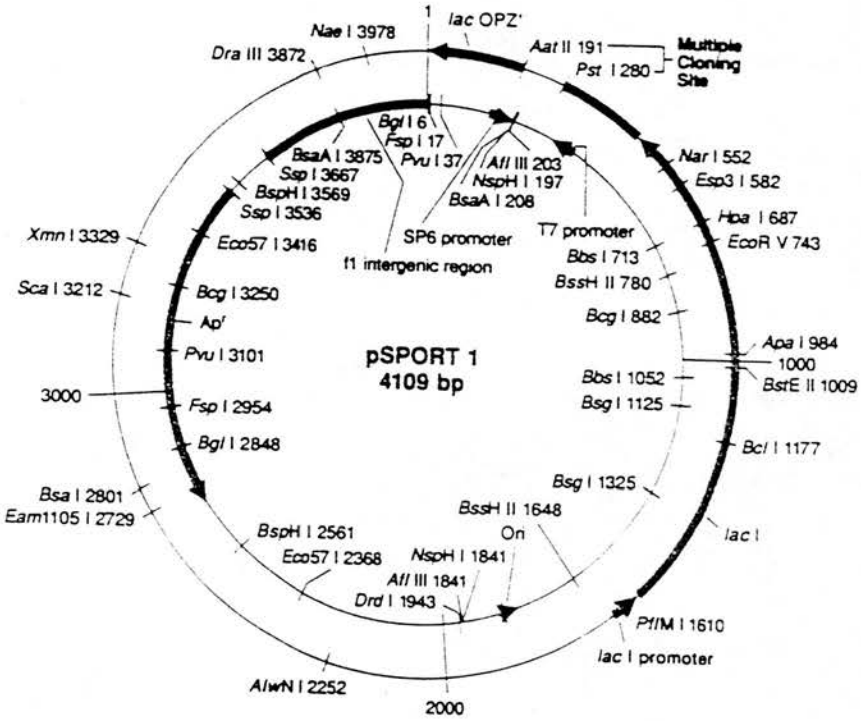


The plasmid DNA was linearised by digestion with NotI so that transcription using the T7 promoter would produce sense RNA. Digestion with SalI and then transcription with the SP6 promoter produced antisense RNA. Transcription with linearised template DNA prevents the additional transcription of pSPORT1 vector sequence i.e. linearisation ensures that only insert sequence which is the probe of interest is transcribed.

Plasmid DNA (10 µg) was mixed with 10x restriction buffer H (5 µl) and NotI or SalI enzyme (30 U) in a total volume of 50 µl. The digests were incubated at 37°C for 2 h. 50 µl of phenol/chloroform (1:1) were then added. After vortexing the tubes were centrifuged at 13,000 g for 5 min at 4°C. The top aqueous layer was removed to a clean Eppendorf tube and 0.1x the volume of 3 M NaAcetate, pH 5.5 and 2x the volume of 100% ethanol were added. After mixing, the tubes were put at -70°C for 30 min and then centrifuged at 13,000 g for 40 min at 4°C. The supernatant was discarded and the pellet washed in 70% ethanol (500 µl). After centrifugation at 13,000 g for 5 min at RT, the supernatant was discarded and the pellet briefly air-dried before it was resuspended in TE buffer (10 µl). It was stored at -20°C at this stage.

Assuming 100% recovery the final linearised plasmid concentration should now be 1 µg/µl. 1 µl of a 1:10 dilution was run out on a 1% agarose electrophoresis gel in 1x TAE buffer against a standard of known concentration to confirm this and

Figure 2.1. Map of DNA plasmid vector pSPORT1 (Gibco) indicating cloning sites.



pSPORT 1

SP6 ↓

Aat II
Sph I
Mlu I
Spl I
SnaB I
Hind III
BamH I
Xba I
Not I
Xma III
Spe I
Sst I
Sal I
Sma I
EcoR I
BspM II
Rsr II
Kpn I
Pst I

T7 ↑

to check that there was no undigested plasmid which could give rise to circular transcription.

2.3.3 Preparation of riboprobes for ISH

The linearised plasmid DNA was transcribed using the SP6/T7 Transcription Kit (Boehringer Mannheim, Cat. No. 999 644). The manufacturers instructions were modified. Linearised DNA (4-5 µg) was incubated with 10 mM ATP, GTP and CTP (1 µl of each), [^{35}S]-UTP (50 µCi) (ICN Biochemicals Ltd.), 10x buffer (2 µl), RNase inhibitor (20 U) and SP6 RNA polymerase (10 U) (to produce antisense RNA) or T7 RNA polymerase (10 U) (to produce sense RNA) in a total volume of 21 µl. The transcription was incubated at 37°C for 30 min. The tubes were then pulse centrifuged before another aliquot of SP6 or T7 RNA polymerase (10 U) was added and incubated at 37°C for another 30 min. RNase-free DNase I (20 U) was added to remove the template DNA. After incubation at 37°C for 15 min and pulse centrifugation, an equal volume of phenol/chloroform (1:1) was added. The tube was vortexed to mix and then centrifuged at 13,000 g for 3 min at RT. The top aqueous layer was removed to a clean Eppendorf tube. The solvent layer was back extracted by the addition of DEPC dH_2O (24 µl). After mixing and centrifuging at 13,000 g for 3 min at RT the top aqueous layer was harvested and combined with the first. The RNA was precipitated by the addition of 0.5x the volume of 7.5 M ammonium acetate, pH 5.5 and 2.5x the volume of cold (-20°C) 100% ethanol followed by 30 min at -70°C.

An aliquot (1 µl) was removed and added to 1 ml of Optiphase 'Hisafe' 3 liquid scintilation cocktail (Wallac). The rest was centrifuged at 13,000 g for 20 min at 4°C. Another aliquot (1 µl) of the discarded supernatant was added to 1 ml of scintilation cocktail. The pellet was washed in 70% ethanol (50 µl), centrifuged at 13,000 g for 5 min at RT and then resuspended in DEPC dH_2O (100 µl). An aliquot (1 µl) of this pellet solution was added to 1 ml of scintilation cocktail.

Next, the riboprobe was reprecipitated overnight at -20°C in 0.1x the volume of 3 M NaAcetate, pH 5.5 and 2x the volume of 100% ethanol. After centrifuging at 13,000 g for 40 min at 4°C, the supernatant was discarded and the pellet washed with 70% ethanol. After counting the radioactivity of the 3 samples (Total, supernatant and pellet), the ammount of incorporated isotope in the transcribed RNA could be calculated and the volume of 0.01 M DTT in which to resuspend the riboprobe determined. Calculation for radioactivity incorporation:-

$$\% \text{ unincorporated } ^{35}S = \frac{\text{unincorporated cpm}}{\text{Total cpm}} \times 100\% = 8.5\% \text{ (e.g.)}$$

Therefore % incorporated ^{35}S = 100% - 8.5% = 91.5%

91.5% of 50 µCi (the initial ammount added) = 45.75 µCi

= 45.75 pMol of UTP

(since 1 µCi = 1 pMol)

1 Mol of dTP = 330 g. Therefore 45.75 x 330 = 15.1 ng

Total RNA = 4 x 15.1 = 60.4 ng

Resuspend the probe at 1 ng/µl/kb

1 ng/µl = 60.4 µl

Riboprobe length = 0.7 kb (e.g.)

Therefore resuspend the probe in 60.4 x $\frac{1}{0.7}$ = 86.3 µl of 0.01 M DTT

2.3.4 Surface labelling of live oligodendrocytes with antibodies before ISH

Coverslips with cultured oligodendrocytes were rinsed briefly in 1x PBS (phosphate buffered saline) made with MilliQ dH_2O. The primary antibody, the monoclonal antibody O4 (Sommer and Schachner, 1981), was diluted (1:10) in 1x PBS and 2% goat serum and applied to and incubated with the cells in a humid chamber for 30 min at RT. The coverslips were then rinsed twice for 2 min in 1x PBS before being fixed with freshly prepared 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4 for 5 min at RT. The coverslips were then washed three times for 5 min in 0.1 M Glycine-HCl in 1x PBS, pH 7.4, and then twice for 5 min in 1x PBS. The coverslips were then glued onto glass slides with Loctite glass to glass glue and exposed to UV light for 10-15 seconds to bond the adhesive. The secondary antibody was then applied (donkey anti-mouse coupled to TRITC, Jackson) diluted (1:50) in 1x PBS and 2% goat serum. The slides were incubated in a humid chamber for 30 min at RT in darkness and then washed twice for 2 min in 1x PBS before continuing with the pre-treatment washes.

2.3.5 Preparation of rat brain sections for ISH

A 21 day old Wistar rat was sacrificed, its brain dissected out and frozen in isopentane at -45°C. Parasagittal cryostat sections of brain (15 µm) were mounted on slides coated with TESPA (3-aminopropyltriethoxysilane) (Sigma) and stored at -70°C.

2.3.6 Pre-treatment washes

The samples were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4 for 5 min at RT. The fixed material was then acetylated for 10 min at

RT (to 50 ml of 0.1 M triethanolamine pH 8, 125 µl of acetic anhydride was added, mixed and quickly poured onto the material). The slides were washed twice for 2 min in 1x PBS (made with DEPC dH_2O) at RT, then for 2 min in 70% ethanol, 2 min in 90% ethanol and twice for 2 min in 100% ethanol. The samples were then air-dried for approximately 30 min.

2.3.7 Hybridisation

The hybridisation buffer consisted of 50% formamide, 10% dextran sulfate, 1x Denhardts, 20 mM Tris-HCl pH 8, 0.3 M NaCl, 5 mM EDTA, 10 mM sodium phosphate pH 8, 0.5 mg/ml yeast tRNA and could be stored in aliquots at -20°C. The riboprobe was diluted 10x with hybridisation buffer containing 10 mM DTT (i.e. 10 µl riboprobe + 0.9 µl 1M DTT + 89.1 µl hybridisation buffer), heated at 80°C for 2 min and then plunged on ice until used. The denatured riboprobe was applied to the sample and covered with a sterile coverslip. Then the slides were placed in a box containing 3MM paper soaked in 50% formamide/5x SSC, the box was sealed and incubated at 50°C overnight.

2.3.8 Post-hybridisation washes

Following hybridisation the slides were taken through a series of washes. The first was in 5x SSC, 10 mM DTT (Wash I) at 50°C for 5 min. This was followed by a wash in 2x SSC, 50% formamide, 0.1 M DTT (Wash II) at 60°C for 20 min. Following this were three washes in 0.5 M NaCl, 10 mM Tris-HCl, 5 mM EDTA (Wash III) at 37°C, each for 5 min. RNAaseA diluted to 20 µg/ml in Wash III was then applied to the slides which were incubated at 37°C for 30 min. The slides were then washed with Wash III at 37°C for 5 min, then Wash II at 65°C for 20 min. This was followed by a 5min wash in 2x SSC at RT and then a 5min wash in 0.1x SSC at RT.

The slides were then taken through a series of washes to dehydrate the samples. The first wash was in 0.3 M NH_4 acetate, 30% ethanol for 2 min at RT. The second wash was in 0.3 M NH_4 acetate, 60% ethanol for 2 min at RT. The third wash was in 0.3 M NH_4 acetate, 80% ethanol for 2 min at RT. The fourth wash was in 0.3 M NH_4 acetate, 95% ethanol for 2 min at RT. These washes were followed by a final wash in 100% ethanol for 2 min at RT. The slides were then air-dried for at least 30 min. At this stage the dried slides could be exposed to X-ray film overnight and the resultant autoradiograph used to estimate the intensity of signal present.

2.3.9 Immunocytochemistry

Immunocytochemistry to label intracellular antigenic markers was done at this stage.

Cultured cells:-

The primary antibody (monoclonal antibody GA5 against GFAP, Boehringer Mannheim) was diluted 1:5 in blocking buffer (1x PBS/1% goat serum/0.1% (v/v) Triton-X-100) and applied to the cells which were then incubated in a humid chamber for 60 min at RT. The slides were washed three times for 5 min in 1x PBS (DEPC) at RT. The secondary antibody (goat anti-mouse coupled to TRITC, Southern Biotech) diluted 1:50 in blocking buffer was then applied and incubated in darkness for 30 min in a humid chamber at RT. The slides were washed three times for 5 min in 1x PBS (DEPC) at RT and then air-dried.

Brain sections:-

Sections were blocked for 2 h at RT in 1x PBS (DEPC)/0.2% pig skin gelatin (BDH) (blocking buffer). The primary antibody (mAb GA5 against GFAP, Boehringer Mannheim) was diluted 1:5 in blocking buffer and applied to the tissue. A coverslip was placed on top and the slides were incubated in a humid chamber overnight at 4°C. The slides were then washed three times for 10 min in blocking buffer before the secondary antibody (goat anti-mouse coupled to TRITC, Southern Biotech) was applied diluted 1:50 in blocking buffer. The slides were incubated in a humid chamber for 2 h at RT in darkness and then washed twice for 10 min in blocking buffer and twice for 10 min in 1x PBS before being air-dried.

2.3.10 Emulsion dipping

An aliquot (6 ml) of a glycerol solution made by mixing glycerol (0.14 ml) and DEPC dH_2O (7 ml) was put in a 50 ml universal tube and warmed to 43-45°C. In a darkroom using only a Kodak orange safe-lamp, emulsion (Nuclear Research Emulsion, Ilford K-5 (A size) 1355127) was added until the volume was 12 ml and then dissolved by warming at 43-45°C for 10-15 min. The solution was transferred to a dipping vessel and then stood in the waterbath for 10 min to allow air bubbles to rise. Each slide was then dipped into the emulsion and the excess wiped off the back. The slides were then left in an upright position for excess emulsion to drain away from the sample and the resultant thin coating to dry (approximately 20 min). The slides were then placed in a box with sachets of autoclaved silica, sealed with tape and wrapped in aluminium foil and black plastic to seal them from the light. The sealed box was left at RT overnight, then at 4°C for 2-3 weeks or as required.

2.3.11 Development

In the darkroom, as for the emulsion dipping, the slides were dipped in D19 (Kodak) developer (made up according to the manufacturers instructions) for 4 min at RT, then in stop solution (1% glycerol/1% glacial acetic acid) for 1 min and then in fix (30% (w/v) thiosulfate) for 3 min. The slides were washed under running tap water for 20 min and then air-dried.

If immunocytochemistry had been done the slides were mounted with anti-fade and the coverslips sealed with nail varnish. Otherwise the samples were counterstained with Ehrlichs heamatoxylin for 20-30 seconds, washed under running water until clear, dipped twice in acid-alcohol, washed under running water for 3 min, dehydrated for 1 min in 70% ethanol, 90% ethanol then 100% ethanol, dipped in xylene for 5 min before being mounted with DePeX mounting medium (BDH) and coverslipped whilst still damp.

2.4 HYBRIDISATION SCREENING OF A CDNA LIBRARY FOR FULL LENGTH CDNA CLONES

2.4.1 Titration of the cDNA library

NZY Broth (5 ml) (Gibco BRL) containing 0.2% maltose and 100 µg/ml ampicillin was innoculated with Y1090-E.coli and incubated at 37°C in an orbital incubator overnight.

The cDNA library analysed was an oligo dT primed, directionally cloned (NotI -> SalI), mixed age (1 day, 15 day and 16 weeks) rat brain library in λgt22A, prepared by K. Rogers. The library phage were diluted 1:100 with SM buffer (0.58 g NaCl, 0.2 g $MgSO_4.7H_2O$, 5 ml 1 M Tris-HCl pH 7.5, 0.5 ml 2% gelatin, dH_2O to 100ml, autoclaved). The diluted phage (1 µl) was added to the Y1090 culture (200 µl) in a 5 ml bijoux and incubated in a 37°C oven for 20 min with swirling every 5 min. 3 ml of Top Agarose (NZY broth + 0.7% agarose) cooled to 48°C were added and mixed by inversion before being poured onto a dry small 90 mm NZY agar plate (NZY broth + 1.5% agar). Once the top agarose had set, the plate was incubated at 37°C overnight. The number of recombinant plaques could then be counted and the titre of the cDNA library established.

2.4.2 Plate preparation for a primary hybridisation screen of the cDNA library

NZY broth (5 ml) containing 0.2% maltose and 100 µg/ml ampicillin was innoculated with Y1090, as above, and incubated overnight at 37°C in an orbital incubator. The cDNA library phage were diluted with SM buffer to give the

appropriate titre required. This required titre e.g. 60,000 plaque forming units (pfu) per 15 cm plate was added to 500 µl of the Y1090 culture and incubated at 37°C for 20 min with swirling every 5 min. NZY Top Agarose (7.5 ml) cooled to 48°C was then added and mixed by gentle inversion before being poured onto a large 15 cm NZY agar plate. Once set, the plate was incubated at 37°C overnight to allow the viral plaques to develop.

2.4.3 Filter lifts

Some of the plaque DNA was blotted onto circular nylon membranes (Duralon-UV membranes, Stratagene) which were layed onto the NZY-agar plates. Orientation marks were made on both the nylon membranes and the agar plates for reference later. The membrane filters were placed, DNA side up, on two layers of 3MM filter paper soaked in denaturation buffer (0.5 M NaOH, 1.5 M NaCl), ensuring the DNA side did not get wet. They were left for 5 min. The filters were then placed onto 3MM paper soaked in neutralising buffer (1.5 M NaCl, 0.5 M Tris-HCl pH 7.4) in the same fashion and left for 2 min. This neutralising step was repeated on fresh 3MM paper and again left for 2 min. The filters were then briefly rinsed in 2x SSC, air-dried and baked in an 80°C oven for 2 h. They could be stored at RT until required.

2.4.4 Hybridisation of filters with:-

a) ^{32}P labelled cDNA probe

The Ready TO GO DNA Labelling Kit (-dCTP) (Pharmacia) was used to radioactively label the cDNA probe required. Plasmid DNA (25-50 ng) boiled for 2 min to denature, was added to the reconstituted kit reaction mix with [α-^{32}P] dCTP (50 µCi) (ICN) and dH_2O to a final volume of 50 µl. The reaction mix was incubated at 37°C for 30 minutes. 100 µl of sheared salmon sperm DNA (10 mg/ml) (Stratagene) was then added to the labelled cDNA probe and boiled for 5 min before being added to prehybridising filters.

Filters were briefly wet with dH_2O and placed in large glass hybridisation tubes which had been coated with dimethyldichlorosilane. Rapid Hyb Hybridisation Buffer (15 ml) (Stratagene) was added to each tube which was then prehybridised at 65°C in a rotary oven for 30 min. On addition of denatured probe the filters were left to hybridise for 1.5 h at 65°C.

The filters were then washed at low stringency twice for 10 min in 2xSSC/0.1%SDS at RT with agitation and twice at high stringency for 30 min in 0.2xSSC/0.1%SDS at 65°C in the rotary oven. They were then allowed to dry

Figure 2.2. Clone OL0755 sequence indicating PCR primers.

11F-> 9F->

5′ GCGAGCTTTGTCCAGACCACCATGGCTCTGGGGCTGCCCTCCAAGAAAGC 50

ATCTTCCCGCAACGTGATCGTGGAGCGCAGGAACCTGATCACCGTGTGCA 100

GGTTCTCTGTGAAAACCTTGCTAGAGAAGTACACAGCAGAACCCATCGAT 150

GATTCATCCGAGGAGTTTGTTAACTTCGCAGCCATTTTAGAGCAGATCCT 200

CAGCCACCGATTTAAAGGTCCAGTGAGCTGGTTCAGCTCAGATGGGCAAC 250

GGGGCTTCTGGGACTATATCCGGCTGGCCTGCAGCAAAGTGCCCAACAAC 300

TGCGTAAGCAGCATCGAGAACATGGAGAACATCAGCACAGCTCGAGCCAA 350

<-7R

GGGCCGGGCGTGGATCCGGGTGGCTCTGATGGAGAAGCGTATGTCAGAAT 400

ACATCACTACAGCTCTTCGGGACAACCGAACTACCAGACGGTTCTATGAC 450

TCCGGAGCCATCATGCTGCGAGAGGAAGCCACTGTCCTCACAGGGATGCT 500

GATCGGACTCAGCGCTATCGACTTCAGCTTCTGTCTAAAGGGCGAAGTTC 550

TGGACGGGAAGACGCCGGTGGTCATCGATTACACACCCTTCAAGAACAAT 600

ACCCAGCTAGCCTCAGGTCCCAGCTAGCCTCGGGTCTCTAGGGAAAGACA 650

ACCTGAGCCTCTTTCCTGCTTCAGCTTCCTGCCAAAGAGGCAGGAGCTAC 700

ATGGGGAAGTGGTGGGGCCAGGAGGGACGCCCAGGTTACAGGTCGTCAGT 750

CCCCTGGGGAAGCCACTCCATTCCGTGGTCCTGAAGGCTGCCTGGTTCCT 800

TCTGTTCATCTTCCACACTTGCCTCAGAAGCAGGTGGTCCAGCCCTGGCA 850

TTCCTGCTGCCCTGCCTCTGGTCTAACCCTGTGTACCCTCTGAAGTCACC 900

CTTCCTCGGTACCTATGTGGGGAGAGATTAGGCA**AATAAA**AACCAGAGG 950

<-9R

ACTGAAAAAAAAAAAAAAAAAAAAAAAA 3′ 1000

slightly before noting the orientation marks with radioactive ink, wrapping them in Saran Wrap and exposing them to X-ray film.

b) Digoxygenin labelled cDNA probe

Primer 9F 5′ AAGCATCTTCCCGCAACGTG 3′

Primer 9R 5′ TGCCTAATCTCTCCCCACATAGGTA 3′

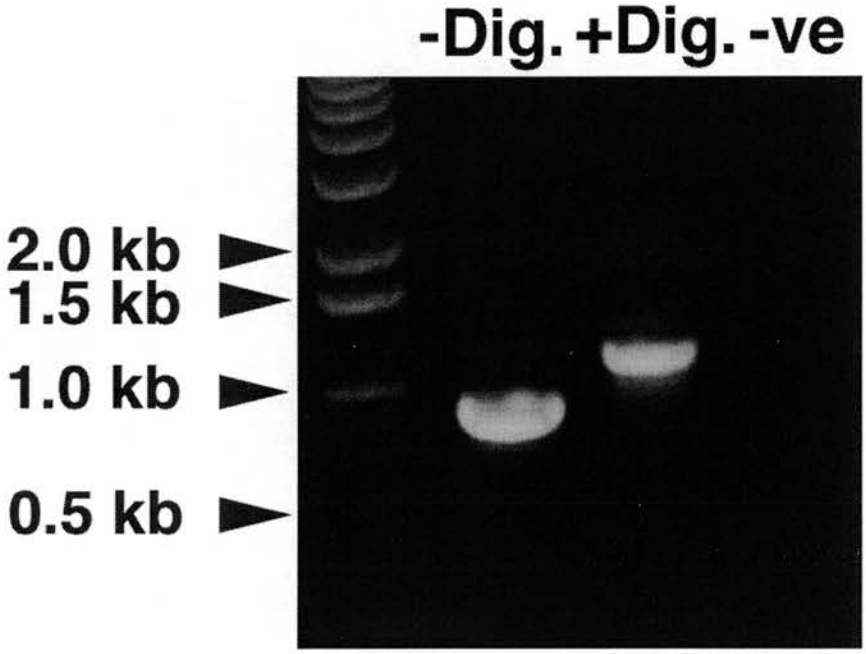
For primer preparation see 2.6.3.

A 904 bp fragment of the 1.1 kb insert for clone OL0755 was amplified by PCR using primers 9F and 9R (see Figure 2.2), incorporating digoxygenein labelled UTP. 10x buffer (5 µl), modified dNTPs (2 mM ATP, GTP, CTP, 1.4 mM TTP) (5 µl), 1 mM digoxygenin-UTP (3.5 µl) (this gives a ratio of 2 UTP : 1 Digoxygenin-UTP), 10 mM primer 9F (5 µl), 10 mM primer 9R (5 µl), plasmid DNA of clone OL0755 diluted 1:500 (1 µl) and Dynazyme DNA polymerase (1 U) were mixed in a total volume of 25 µl and overlaid with mineral oil (25 µl). A control PCR reaction was set up in the same way, except with regular 2 mM dNTPs. Amplification conditions were:- 29 cycles of denaturation at 94°C for 40 seconds, annealing at 59°C for 1 minute and extension at 72°C for 40 seconds, followed by a final cycle of denaturation at 94°C for 40 seconds, annealing at 59°C for 1 minute and extension at 72°C for 7 minutes. The PCR reactions were then run out on a 1% agarose electrophoresis gel in 1x TAE buffer. Digoxygenin incorporated during amplification retards mobility of the product during electrophoresis as shown in Figure 2.3, so comparison with the control reaction indicates how successful the reaction was.

The digoxygenin incorporated PCR product bands were cut out of the gel and the amplified DNA purified using the Qiaex II gel extraction kit (see 2.5.1 for details). The purified DNA was eluted first in 30 µl and then in 20 µl (total volume of 50 µl). An aliquot was run on a gel against a standard of known concentration to estimate the concentration of the digoxygenin labelled probe. The probe (2.5 µg) diluted to 50 µl with TE buffer was mixed with 50 µl of sheared salmon sperm DNA (10 mg/ml) and denatured at 98°C for 10 min before being added to prehybridising filters (see 2.4.4a). Filters were left to hybridise for 1.5 h at 65°C, then washed at low stringency twice for 10 min in 2xSSC/0.1%SDS at RT with agitation and twice at high stringency for 30 min in 0.2xSSC/0.1%SDS at 65°C in the rotary oven.

Positive plaques were visualised by first washing the filters for 5 min at RT in 10 ml of Buffer I (0.1 M maleate, 0.15 M NaCl, pH 7.5) with gentle shaking. They were then incubated with 10 ml of Buffer II (1% Boehringer blocking agent in Buffer I, w/v) for 40 min at RT with agitation, followed by incubation for 40 min at

Figure 2.3. Digoxigenin incorporation during PCR amplification of clone OL0755 with primers 9F and 7R. The incorporation of digoxigenin causes a retardation in mobility of the amplified product on a 1% agarose electrophoresis gel as can clearly be seen. The negative control with no added template DNA is clear.


-Dig.
+Dig.
-ve
2.0 kb
1.5 kb
1.0 kb
0.5 kb

RT in 5 ml of anti-Digoxygenin antibody-AP diluted 1:5000 with Buffer II. Filters were washed twice for 5 min in 10 ml of Washing Buffer (0.3% Tween 20 in BufferI) followed by 2 min in 10 ml of freshly prepared Buffer III (0.1 M Tris-HCl, 0.1 M NaCl, 0.05 M $MgCl_2$, pH 9.5). They were then left in 3 ml of freshly prepared colour solution (5 ml Buffer III + 25 µl NBT (50 mg/ml, Promega) + 19 µl BCIB (50 mg/ml, Promega)) in darkness for the colour reaction to develop. The filters could then be washed under tap water, dried and stored.

Individual plaques containing DNA which had hybridised with the probe were identified. Plugs were removed from the NZY-agar plates containing the positive plaques of interest with a cut-off yellow pipette tip and placed in 1.5 ml Eppendorf tubes with SM buffer (500 µl) and chloroform (50 µl). These were then left at RT for at least 4 h to lyse the Y1090 bacteria. The resultant lysate could then be re-titrated as before (See 2.4.1)

A secondary hybridisation screen could be done but with fewer pfu per small 90 mm NZY-agar plate (approximately 200) which can be used for taking filter lifts etc. In this way positive plaques of interest are diluted and purified. After 2 or 3 rounds of hybridisation screening it was possible to cleanly isolate a plug from an NZY-agar plate with a single pure plaque containing a single cDNA clone from the library.

2.4.5 PCR amplification of plug lysates to confirm the presence of clone OL0755 sequence

Primer 11F 5´AGCTTTGTCCAGACCACCAT3´

Primer 7R 5´GGCTCGAGCTGTGCTGAT3´

For primer preparation see 2.6.3.

10x buffer (5 µl), 2 mM dNTPs (5 µl), 5µM primer 11F (5µl), 5µM primer 7R (5µl) and lysate(1 µl) (template) in a total volume of 49.5 µl was overlaid with mineral oil (50 µl) and heated to 94°C before the addition of Dynazyme DNA polymerase (1 U). A negative control was prepared with no added template DNA. Amplification conditions were:- 29 cycles of denaturation at 94°C for 40 seconds, annealing at 65°C for 1 minute and extension at 72°C for 40 seconds followed by a final cycle with extension for 7 minutes. An aliquot (10 µl) of the reaction mix was run out on a 1% agarose electrophoresis gel with a 1 kb size marker. A band of about 300 bp indicates the amplification of clone OL0755 sequence.

2.4.6 PCR amplification of plug lysates to estimate extra 5´ sequence

Primer 601 5´GGCACATGGCTGAATATCGA3´

Primer 7R 5´GGCTCGAGCTGTGCTGAT3´

For primer preparation see 2.6.3.

10x buffer (5 µl), 2 mM dNTPs (5 µl), 5 µM primer 601 (5 µl), 5 µM primer 7R (5 µl) and lysate (1 µl) (template) in a total volume of 49.5 µl was overlaid with mineral oil (50 µl) and heated to 94°C before addition of Dynazyme DNA polymerase (1 U). A negative control was prepared with no added template DNA. Amplification conditions were:- 29 cycles of denaturation at 94°C for 40 seconds, annealing at 62°C for 1 minute and extension at 72°C for 1 minute and 40 seconds followed by a final cycle with extension for 7 minutes. An aliquot (10 µl) of the reaction mix was run out on a 1% agarose electrophoresis gel with a 1 kb size marker to try and estimate how much extra 5´ sequence, if any, had been amplified.

2.4.7 Preparation of high titre lysates for purified plaques of interest

After titration of the lysate of the purified plaque of interest, 50,000 pfu per small 90mm NZY-agar plate were allowed to grow to confluence at 37°C overnight (see 2.4.2). The plates were then flooded with SM buffer (4 ml) and left at RT with gentle agitation for at least 6 h. The resultant lysate was removed, aliquoted in fractions (1 ml) with chloroform (50 µl) and stored at 4°C until required.

2.4.8 Isolation of plasmid DNA

Two aliquots (1 ml each) of high titre lysate were centrifuged at 13,000 g for 5 min at RT, the third was kept as a backup. The supernatant was removed to a clean Eppendorf tube and 350 µl of 30% PEG (polyethylene glycol) in 3 M NaCl were added. The tubes were mixed and left on ice for 30 min. After centrifugation at 13,000 g for 10 min at 4°C the supernatant was discarded. After re-centrifugation at 13,000 g for 2 min the residual supernatant was discarded. Both pellets were then resuspended together in 250 µl of filter sterilised PSB (10 mM Tris-HCl pH 7.5, 10 mM MgSO4, 100 mM NaCl) by gentle pipetting. 5 µl of RNAase cocktail (1 mg/ml RNaseA, 20,000 U/ml RNaseT1; Ambion) and 1.5 µl of DNAaseI (1 mg/ml in H_2O) were added, mixed and then incubated at 37°C for 30 min. Then 250 µl of 0.3 M Tris-HCl pH 9, 100 mM EDTA, 1.25% SDS (freshly made) were added, mixed and incubated at 65°C for 10 min. 250 µl of solution P3 (See 2.3.1) were then added, mixed and left on ice for 5 minutes. After centrifuging at 13,000 g for 2 min at 4°C, the supernatant was removed to a clean Eppendorf tube and an equal volume of phenol/chloroform (1:1) was added. Following mixing the tube was centrifuged at

13,000 g for 5 min at RT. The upper aqueous phase was removed to a clean Eppendorf tube and the phenol/chloroform extraction repeated. 0.8x the volume of isopropanol was added to the isolated aqueous phase, mixed and left on ice for 10 min. The tube was then centrifuged at 13,000 g for 5 min at 4°C. The supernatant was discarded and the pellet washed with 70% ethanol (0.5 ml). Following centrifugation at 13,000 g for 5 min at RT the pellet was resuspended in TE buffer (20 µl).

An aliquot (3 µl) of the purified plasmid DNA could then be digested at 37°C with NotI (5 U) and SalI (5 U) in a volume of 10 µl with 10x restriction buffer H (Boehringer) and run out on a 1% agarose electrophoresis gel in 1x TAE buffer to confirm the presence and size of an insert.

2.5 EXCISION OF INSERT DNA AND LIGATION INTO A SUITABLE VECTOR FOR SEQUENCING

2.5.1 Purification of excised DNA using QIAEX II Gel Extraction Kit

The released insert DNA from the digested plasmid was cut out of a 1% agarose gel and purified using the QIAEX II Gel Extraction Kit (Qiagen). Buffer QX1 was added to the gel fragment in an Eppendorf tube at 3x the volume of the gel for DNA fragments of 100 bp-4 kb e.g. 300 µl of buffer QX1 was added to 100 mg of gel. Resuspended QIAEX II (10 µl) was then added and the tube was vortexed before incubation at 55°C for 10 min. Mixing was repeated every 2 min to keep the QIAEX II in suspension. Following centrifugation of the sample at 13,000 g for 30 seconds at RT, the supernatant was discarded. The pellet was washed with buffer QX1 (0.5 ml) to remove agarose contaminants and then centrifuged at 13,000 g for 30 seconds at RT. The supernatant was discarded and the pellet washed twice with buffer PE (0.5 ml) to remove salts, centrifuging at 13,000 g for 30 seconds at RT each time. The pellet was then left to dry for 10-15 min, then resuspended in TE buffer (15 µl) by vortexing. It was left at RT for 5 min to elute the DNA and then centrifuged at 13,000 g for 30 seconds at RT. The supernatant containing eluted DNA was removed to a clean Eppendorf tube. Elution of DNA from the QIAEX II pellet was repeated by the addition of another aliquot of TE buffer (5 µl). Incubation at RT, centrifugation and removal of the supernatant was repeated as before and the two eluants were then combined (15 µl + 5 µl).

2.5.2 Ligation of purified DNA

The purified NotI/SalI digested insert DNA was ligated into a suitable plasmid for sequencing e.g. pSPORT I (Gibco). The purified DNA (20 µl) was mixed with 5x T4 ligase buffer (6 µl), T4 ligase (2 U) and NotI/SalI digested pSPORT I plasmid (10 ng) in a total volume of 29 µl. The reaction was incubated at 21°C overnight.

2.5.3 Preparation of XL-1 Blue competent cells

Sterile L-Broth (5 ml) containing tetracycline (5 µg) was inoculated with XL-1 Blue E.coli bacteria and incubated overnight at 37°C in an orbital incubator (220 rpm).

The culture was diluted 1:50 with L-broth (5 ml) and then incubated at 37°C in the orbital incubator for 3 h until the optical density (O.D.) reached 0.4-0.7. The culture was then centrifuged at 2000 rpm for 10 min at RT. The supernatant was discarded and the pellet gently resuspended in cold, sterile 0.1 M $CaCl_2$ (2.5 ml). This was left on ice for 20 min with occasional shaking. The cells were then centrifuged at 2000 rpm for 10 min at RT, the supernatant discarded and the pellet resuspended in cold 0.1 M $CaCl_2$ (0.5 ml).

2.5.4 Transformation of ligated plasmid into competent XL-1 Blue E.coli

To 100 µl of competent cells the entire ligation mix was added. This was then incubated on ice for 30 min with occasional shaking. The cells were heat shocked at 42°C for 2.5 min, added to L-Broth (5 ml) and incubated at 37°C in the orbital incubator (220 rpm) for 1 h. Following centrifugation for 10 min at 2000 rpm at RT most of the supernatant was discarded before the pellet was resuspended in a volume of 50 µl and spread on X-Gal agar plates. These were prepared from L-broth (500 ml) containing agar (7.5 g), ampicillin (50 mg), 4% X-Gal (1 ml) and 1 M IPTG (150 µl). The plates were incubated overnight at 37°C. White colonies indicated recombinant bacteria containing plasmids with inserts of interest. Insertion of new DNA inactivated β-galactosidase synthesis. X-gal is cleaved by β-galactosidase into a product which is bright blue. Since the recombinants did not have β-galctosidase their plaques appeared colourless (white). These colonies were picked from the plate and cultured in L-broth (5 ml) containing ampicillin (5 mg) by incubation at 37°C overnight in the orbital incubator (220 rpm).

2.5.5 Alkaline lysis to purify the plasmid DNA

An aliquot (0.5 ml) of the culture was mixed with an equal volume of 50% glycerol (sterile) and stored as a stock at -70°C.

Another aliquot (1.5 ml) of the culture was centrifuged at 13,000 g for 1 min at RT and the supernatant discarded. The pellet was resuspended in cold solution P1 (See 2.3.1) by vortexing. Freshly prepared solution P2 (See 2.3.1) (200 µl) was added and mixed by inversion. Cold solution P3 (See 2.3.1) (150 µl) was added and mixed by vortexing before being left on ice for 5 min. Following centrifugation at 13,000 g for 5 min at 4°C the supernatant was removed to a clean Eppendorf tube and an equal volume of phenol/chloroform (1:1) was added. The tube was vortexed and then centrifuged at 13,000 g for 5 min at RT. Addition of 0.1x the volume of 3M Na acetate, pH 5.5 and 2x the volume of 100% ethanol to the isolated aqueous phase was followed by 30 min at -70°C. The tube was then centrifuged at 13,000 g for 40 min at 4°C and the supernatant discarded. The pellet was washed in cold 70% ethanol (0.5 ml) and then centrifuged at 13,000 g for 5 min at RT. After being briefly air-dried the pellet was resuspended in TE buffer (20 µl) containing RNAase cocktail (2 µl) (1 mg/ml RNaseA, 20,000 U/ml RNaseT1; Ambion).

An aliquot (1 µl) of the purified plasmid DNA was digested with NotI (5 U) and SalI (5 U) with 10x buffer H in a final volume of 10 µl at 37°C and then run out on a 1% agarose electrphoresis gel in 1x TAE buffer to confirm the presence of the insert DNA of interest, before sequencing.

2.6 SEQUENCING OF PLASMID DNA

2.6.1 Sample preparation for sequencing

Template DNA (1.5-2 µg) was taken to a volume of 12 µl with MilliQ dH_2O in an Eppendorf tube. 2 M NaOH (3 µl) was added and the mixture left to incubate at RT for 10 min. 3 M Na acetate, pH 4.7 (4.5 µl) and MilliQ dH_2O (7 µl) were then added and mixed followed by 100% ethanol (90 µl). The tube was put at -70°C for 20 min and then centrifuged at 13,000 g for 10 min at 4°C. The supernatant was discarded and the pellet washed with cold 70% ethanol (100 µl). Following centrifugation at 13,000 g for 2 min at RT, the supernatant was discarded and the pellet air-dried before being resuspended in MilliQ dH_2O (5 µl).

The Sequencing Kit (Pharmacia) was used. Annealing buffer (1 µl) and 1µl of the required primer (5 µM) were added to the resuspended pellet. This reaction was incubated at 37°C for 20 min and then at RT for 10 min. Meanwhile four tubes labelled A, G, T and C were set up with A mix, G mix, C mix and T mix

respectively (1.5 µl). The T7 DNA polymerase (0.5 µl) was diluted with dilution buffer (1 µl) and TE buffer (1 µl) (NB this is enough for 2 DNA templates). To each tube containing template DNA and primer, Labelling mix A (1.5 µl) was added with [^{35}S]-ATP (0.2 µl), the diluted T7 polymerase (1 µl) and dH_2O (0.3 µl) (Total volume of 10 µl). This reaction mixture was incubated at RT for 4 min. The DNA template mixture (2.3 µl) was then added to each of the A, G, T and C mix tubes which were then incubated at 39°C for 5 min. Stop solution (2.5 µl) was added to each and the samples were stored at -20°C for up to 1 week.

2.6.2 Sequencing gel

A 6% acrylamide, 42% urea (w/v), 1x TBE (Tris-HCl, boric acid, EDTA pH 8) 60cm gel was run in 1x TBE at 2900 V and 70 W for 30 minutes to warm up. Once all the samples had been loaded, the gel was run at 2900 V and 60 W until the desired separation of bands had occured (usually around 3.5 h).

The gel was fixed in 5% methanol/5% acetic acid in dH_20 for 20-30 min before being transferred onto 3MM filter paper and dried under vacuum at 80°C for 1 h. Once dry, the gel was exposed to X-ray photographic film. From the resultant autoradiograph the sequence of the DNA could be read.

2.6.3 Primer preparation

Primers were synthesised on the PCR Mate Oligonucleotide Synthesiser on appropriate columns (A, G, T or C columns depending on the desired 3´ nucleotide). Recovery and purification of the synthesised primer must first involve detachment from the column. The column was flushed with ammonium hydroxide (0.6 ml) and left in this solution at RT for 20 min before draining into a small bijoux bottle. This was repeated twice more to produce a total volume of 1.8 ml of primer and ammonium hydroxide solution. This solution was incubated at 56°C overnight to complete the breakdown of side chain protecting groups.

An aliquot (200 µl) of the primer/ammonium hydroxide solution was put into a 1.5 ml Eppendorf tube with butanol (1 ml). The tube was vortexed and then centrifuged at 13,000 g for 2 min at RT. The supernatant was removed. Another aliquot (200 µl) of primer/ammonium hydroxide solution and butanol (1 ml) were added to the pellet. The mixture was vortexed and centrifuged as before. This was continued until all the primer/ammonium hydroxide solution had been processed and the primer had been precipitated. The pellet was gyrovapped to remove all traces of solvent and then resuspended in MilliQ dH_2O (0.5 ml).

Spectrophotometric measurements (LKB-Ultrospec III, Pharmacia Biotech) of the primer solutions were used to calculate concentration. Readings were done on aliquots (2 µl) of primer solution diluted 500 times with MilliQ dH_2O to a final volume of 1ml in the cuvette. The optical density (O.D.) was measured at 260 nm to estimate nucleic acid content. The following formulae were used to calculate concentration and molarity:-

$$\frac{33 \times \Delta 260\ nm}{2} = \mu g/\mu l$$

$$\frac{\mu g/\mu l}{MW} = \text{Molarity}$$

The molecular weight (MW) of the primer was calculated from its sequence. The sum of the molar values for each nucleotide was divided by 1.06. The molar values used were 331.2 for A, 307.2 for C, 347.2 for G and 306.2 for T.

2.7 RAISING A POLYCLONAL ANTIBODY AGAINST A SYNTHETIC PEPTIDE

2.7.1 Dialysis of KLH (Keyhole Limpet hemocyanin) solution

A 30 cm length of dialysis tubing was boiled for 5 min in MilliQ dH_2O containing a small amount of $NaHCO_3$ and EDTA. The water was allowed to cool before the tubing was thoroughly rinsed in fresh MilliQ dH_2O. One end of the tubing was knotted twice and 6 ml of KLH solution (10 mg/ml) placed inside. The tubing was closed with a single knot, placed in 2.5 L of 1x PBS at 4°C and left to dialyse for 2 h. The PBS solution was then replaced with fresh and dialysis continued at 4°C overnight. The PBS solution was then replaced with 10 mM potassium phosphate, pH 7 and the dialysis continued for a further 6 h at 4°C. The dialysed KLH was then removed from the tubing to a 15 ml Corex centrifuge tube and centrifuged at 8500 rpm for 10 min. The supernatant was removed to a 20 ml Universal tube.

An aliquot (100 µl) of the KLH solution was diluted to 1 ml with dH_2O and its absorbance at 280 nm measured. This gives an ODU per 100 µl, therefore multiplying this reading by 10 gives an ODU per ml. Using the standard of 18 ODU = 10 mg/ml the concentration of the KLH solution was calculated from the ODU reading.

The dialysed KLH solution was divided into two aliquots (3.5 ml each) and stored at -45°C.

2.7.2 Preparation of a Sephadex G-50 chromatography column

12 g of G-50 Sephadex (Sigma) was swollen at RT for 30 minutes in 0.1 M potassium phosphate, pH 6. An excess volume of potassium phosphate was added to the unswollen sephadex, mixed by swirling and then left to sediment. The mixture was swirled and left to sediment again before the buffer solution was poured off and replaced. This process of swirling to mix and sedimentation followed by replacing of the buffer was repeated a further three times to ensure the removal of all loose debris. The swollen Sephadex was then poured into a clamped glass column (19 cm long, 4 cm diameter) and allowed to settle. The column was equilibriated with 0.1 M potassium phosphate, pH 6 (100-150 ml) before being stored at 4°C until required.

2.7.3 Preparation of MBS activated KLH

12.5 mg of sulfo-MBS (maleimidobenzoic acid N-hydroxysuccinimide) was dissolved in 10 mM potassium phosphate, pH 7 (2.5 ml) in a 20 ml Universal tube. When dissolved 3.75 ml (23.85 mg) of dialysed KLH was added and the tube mixed on a rotating windmill for 30 min at RT. This reacted sulfo-MBS-KLH was then applied to the prepared G-50 Sephadex column and eluted with 0.1 M potassium phosphate, pH 6. After the first 20 ml had eluted through the column fractions (1.5 ml) were collected and their absorbance at 280 nm measured. Six fractions were pooled together with an average OD/ml = 1.563 and stored at -40°C.

2.7.4 Conjugation of a peptide to activated KLH

A peptide (20 mg) synthesised by Professor Nigel Groome, Oxford Brookes University, was dissolved in 10 mM HCl (2 ml) in a 15 ml glass tube. An equal volume of ether was added, mixed gently and the mixture allowed to separate into two phases. The lower phase containing the peptide was transferred to a fresh Universal tube and the pH adjusted to 6 by the dropwise addition of 0.1 M NaOH. 1.5 ml of each peptide was then added to 2.25 ml of activated KLH on ice and mixed. The tube was then mixed by rotation on a windmill at 4°C overnight.

2 ml of 0.5 M ethanolamine, pH 7 was added and rotation on the windmill continued for 1 h at 4°C. The conjugated KLH-peptide was then dialysed twice against 2 L of 1x PBS before storage at -45°C.

2.7.5 Innoculation of rabbits with KLH-peptide

The KLH-peptide (1.5 ml) was emulsified overnight with Freunds complete adjuvant (2 ml) with shaking at 4°C. Two New Zealand White rabbits were

innoculated with 0.5 ml in each hind limb and 0.75 ml subcutaneously (Total of 1.75 ml per rabbit).

After 10 days test bleeds were taken. Blood (2-5 ml) was taken from each animal and placed in a Universal tube. It was left at RT for a few hours to begin to clot. Ringing with a stick allowed the resultant blood clot to contract away from the walls of the tube. It was left at 4°C overnight. The resultant serum was then removed to a 15 ml Corex tube and centrifuged at 8500 rpm for 15 min. The supernatant was aliquoted and stored at -45°C.

Rabbits either received booster injections or were sacrificed by cardiac puncture under anaesthesia. The blood collected (usually 50-100 ml) was allowed to clot, ringed and centrifuged etc. as for the test bleed. The antisera were tested by Western blotting.

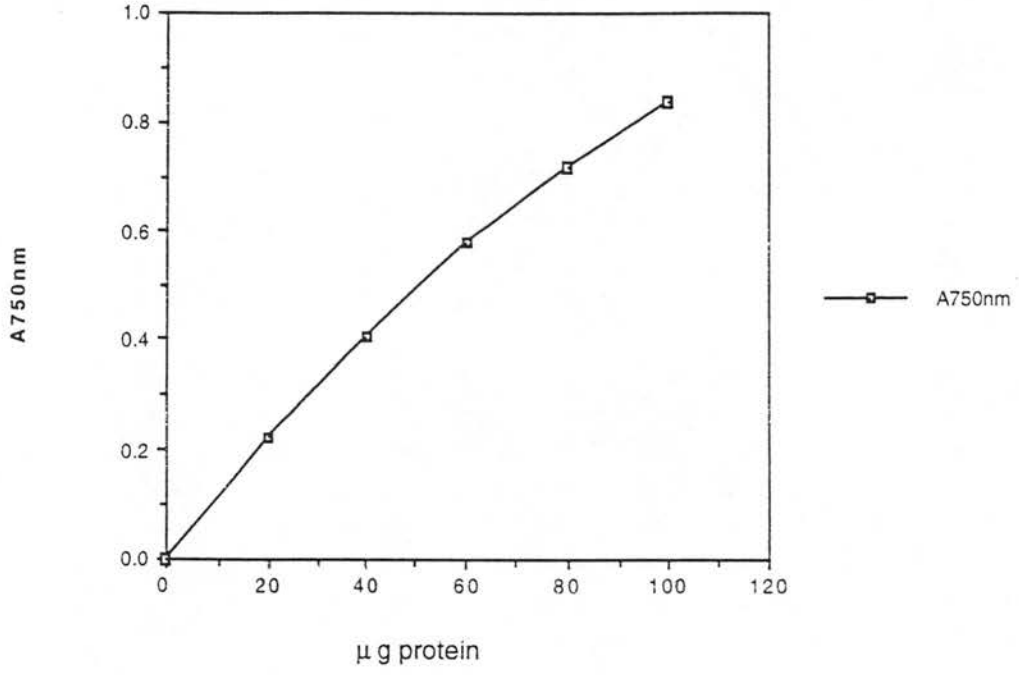
2.8 WESTERN BLOTTING

2.8.1 Preparation of rat brain homogenates for Western blotting

Wistar rats were sacrificed by lethal injection and the brains rapidly dissected out. A small portion of each brain (approximately 0.25 cm^3 in size) including some tissue from the cerebellum and cerebral hemispheres was homogenised in sterile 1x PBS/0.1% Triton-X-100 (2 ml). The homogenate was then aliquoted and stored at -40°C.

A Lowry Test was done on the brain homogenates to determine protein concentration. Reactions were performed in 2 ml tubes in duplicate. 0, 10, 20, 40, 60, 80 and 100 µg of BSA (Bovine serum albumen) standard samples were made up to 100 µl with 1x PBS/0.1% Triton-X-100 (since this is the buffer in which the rat brain tissue samples were homogenised). The brain homogenates to be measured were diluted 1:10 with 1x PBS/0.1% Triton-X-100 in a final volume of 100 µl. Freshly prepared reagent A (1 M NaOH/0.25% SDS) (125 µl) was added to each, mixed and incubated at 60°C for 15 min. Freshly prepared reagent B (250 µl 2% K, Na tartrate; 250 µl 1% $CuSO_4$; 24.5 ml 2% Na_2CO_3) (1.25 ml) was added to each, mixed and left at RT for 15 min. 125 µl of Folin reagent (which had been diluted 1:1 with dH_2O) was then added to each reaction, mixing immediately and then left on ice for 45 min. The absorbance at 750 nm was measured for each sample. A reference curve was constructed by plotting the absorbance readings of the standard BSA samples against their protein concentrations. From this standard curve and their absorbance readings the protein concentrations for each brain homogenate was deduced. (see Figure 2.4)

Figure 2.4. Lowry Test standard curve constructed by plotting absorbance readings at 750 nm of standard BSA samples against protein concentration (μg). Using this curve the protein concentrations of rat brain homogenates were deduced from their absorbance readings.


A750nm
1.0
0.8
0.6
0.4
0.2
0.0
0
20
40
60
80
100
120
µ g protein
A750nm

2.8.2 SDS Polyacrylamide Gel Electrophoresis (SDS PAGE)

A 10% polyacrylamide denaturing gel was prepared:-

13.3 ml 30% stock acrylamide

10 ml 4x stock solution IIA (1.5 M Tris-HCl, pH 8.9)

21.4 µl TEMED

202 µl 20% SDS

150 µl 10% ammonium persulfate (w/v). Freshly prepared.

dH_2O to a final volume of 40 ml.

A 10 cm x 12 cm slab gel was poured in a vertical position. Butanol (0.5 ml) was overlayed on the top surface of the gel to maintain a level surface. Once the gel had polymerised, the butanol was rinsed away with dH_2O. A stacking gel was then poured on top:-2.25 ml 30% acrylamide

1.875 ml 5.7% Tris-HCl, pH 6.7 (w/v)

75 µl TEMED

150 µl 10% ammonium persulfate (w/v)

dH_2O to a final volume of 15 ml.

A 15 well comb was inserted and the gel allowed to polymerise.

Brain homogenate samples for loading were prepared in a volume containing 1x sample buffer (3x stock = 20% glycerol, 2.28% Tris-HCl pH 6.7 (w/v), 8% SDS) and 67 mM DTT. They were boiled for 5 min to denature the proteins then loaded onto the gel together with pre-stained SDS molecular weight markers (27,000-180,000 MW, Sigma). The gel was run in 1x running buffer (25 mM Tris, 250 mM glycine pH 8.3, 0.5% SDS) at 30 mA for 3-4 h. (20x stock running buffer = 15g tris, 72 g glycine, dH_2O to 500 mls, pH 8.4).

2.8.3 Preparation of filters for Western blotting

Once the SDS PAGE gel had run sufficiently to separate the proteins it was removed from the glass plates and the proteins transferred onto a nitrocellulose membrane (Protran Nitrocellulose BA85 0.45 mm Nucleic acid and protein transfer membrane, Schleicher and Schuell) in 20% methanol/2x running buffer (see 2.8.2) at 0.5 A for 1.5 h. The transfer was then placed in blocking buffer (0.2% gelatin (w/v), 0.1% Triton-X-100 (v/v), 1x PBS) for 3 h at RT. It was then dried and stored between two sheets of 3MM filter paper until required. The gel was stained with Comassie Blue and destained in 45% methanol/10% glacial acetic acid to determine how successful transfer had been.

2.8.4 Western blotting of filters

The filter was wet with 1x PBS briefly, then washed twice for 5 min in blocking buffer (see 2.8.3) with agitation. The primary antibody (Ab755) diluted 1:500 in blocking buffer was applied and incubated with the filter at RT for 1 h with agitation. The filter was then washed 3x for 5 min in blocking buffer with agitation. The secondary antibody conjugated to horseradish peroxidase (donkey anti rabbit HRP) was applied at an approriate dilution (1:500) in blocking buffer and left to incubate with the filter for 1 h at RT with agitation but in darkness. The filter was washed 3x 5 min in blocking buffer then for 5 min in 1x PBS. A solution of DAB (diaminobenzidine) dissolved in 50 mM Tris-HCl pH 7.4 (1 mg/ml) was applied to the filter and 1-2 µl of hydrogen peroxide per ml of DAB solution was added to activate a colour reaction allowing visualisation of proteins which had reacted with the primary antibody. SDS solution (20%) was added to stop the reaction when sufficient. The filter was washed in tap water, dried and stored.

2.9 DEPHOSPHORYLATION OF PROTEINS

To 100 µg of brain homogenate, 10x buffer (5 µl), 20 mM $MnCl_2$ (5 µl), BSA (0.5 µg) and a 10x cocktail of protease inhibitors (5 mM PMSF, 0.01 mg/ml antipain, 0.05 mg/ml chymostatin, 20 mg/ml TLCK and 0.01 mg/ml leupeptin) (5 µl) were added in a final volume of 50 µl. To this mixture λ phosphatase (800 U) (New England Biolabs) was added, mixed and incubated at 30°C for 30 min. A control reaction was set up with all the same reagents except no phosphatase was added. 3x sample buffer (25 µl) (see 2.8.2) and 1 M DTT (5 µl) were then added. The samples were boiled and run on a 10% SDS PAGE gel. The proteins were transferred onto a nitrocellulose membrane and incubated with Ab755 at a 1:500 dilution as a Western blot (see 2.8.4).

2.10. DEGLYCOSYLATION OF PROTEINS

To 300 µg of brain homogenate 3 µl of 20% SDS (final 1%), 3 µl of 20% mercaptoethanol (final 1%) and 10x protease inhibitors cocktail (see 2.9) were added in a final volume of 60 µl. This mixture was boiled for 2.5 min. To it were added 12 µl of 0.5 M EDTA (final 0.02 M), 30 µl of 10% Triton-X-100 (final 1%), 30 µl of 1 M phosphate buffer pH 6.3 (final 0.1 M), 30 µl of 10x protease inhibitors cocktail (see 2.9) and N-Glycosidase F (1.2 U) (Boehringer Mannheim) in a final volume of

300 µl. The reaction was mixed and left at RT overnight. A control reaction was set up with the same reagents but no enzyme was added.

Since the maximum possible volume loaded onto an SDS PAGE gel is approximately 150 µl it was necessary to concentrate the samples by precipitation first. The samples were placed on ice and 30 µl of dH_2O were added. Then 30 µl of 0.18% sodiumdeoxycholate (final 0.015%) and 120 µl of 24% TCA (final 6%) were added to give a final volume of 480 µl. This was mixed and left on ice for 15-20 min to precipitate the proteins. After centrifugation at 13.000 g for 5 min at RT the supernatant was discarded and the pellet washed with 6% TCA (1 ml). After further centrifugation at 13,000 g for 5 min at RT the supernatant was discarded and the pellet washed with cold acetone (-20°C) (1 ml). Again the sample was centrifuged at 13,000 g for 5 min at RT, the supernatant discarded and the pellet allowed to dry at 37°C for 15-20 min. The pellet was resuspended in dH_2O (50 µl) and added to it were 3x sample buffer (25 µl) (see 2.9) and 1 M DTT (5 µl). Dropwise addition of 1 M Tris-HCl pH 7.5 brought the acidic pH (yellow in colour) back to neutral (just turned blue again). Approximately 10 µl was required. The samples were then boiled and loaded onto a 10% SDS PAGE gel. Transfer of the proteins onto a nitrocellulose membrane was followed by Western blotting with Ab755.

CHAPTER 3. RESULTS

3.1 Identification of novel nervous system specific cDNA clones

As a prelude to the project, a number of cDNA clones were isolated from a differentiated glial subtractive library with a complexity of approximately 300,000. Those plasmids containing insert DNA fragments which were larger than 0.6 kb were partially sequenced. Comparisons with the Genbank and EMBL databases identified the cDNA clones which had no sequence counterparts in the databases. At the time this project was initiated, the characterisation of the clones can be summarised as below :-

Total number of isolated clones analysed	1003
Number of clones with inserts < 0.6 kb	667
Number of clones with inserts ≥ 0.6 kb and therefore partially sequenced	336
Number of ribosomal sequences identified	170
Number of known sequences identified	80
Number of novel sequences identified	86

Many of the known sequences identified included well characterised clones such as those encoding the myelin basic proteins and proteolipid proteins (see Table 3.1) Their presence indicated that the subtraction process had been successful in isolating clones specific to differentiated glial cells. Bacterial sequences and cloning artefacts were also identified.

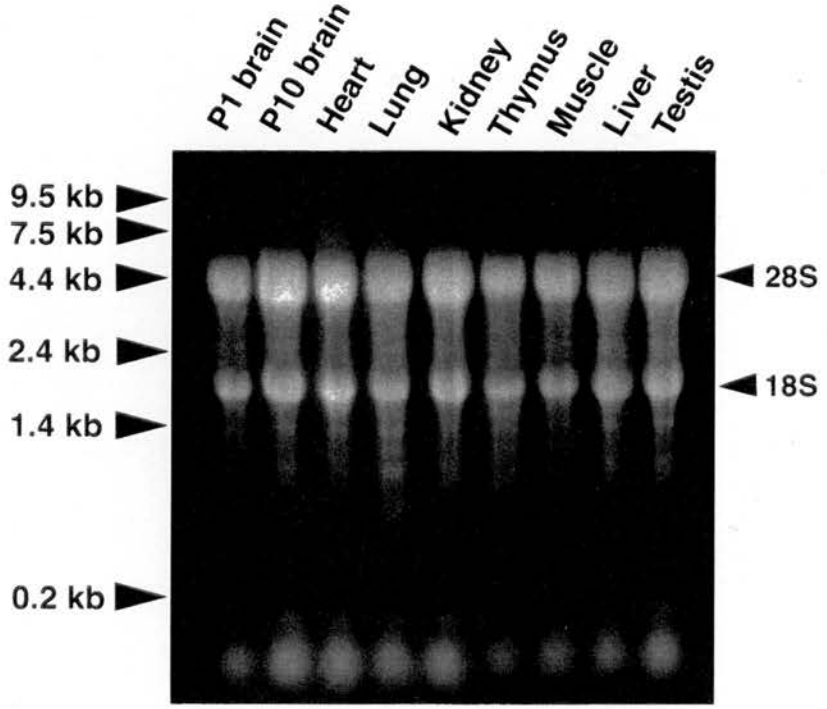
The next stage of selection was to identify which of the novel cDNA clones had a pattern of expression specific to the nervous system. This was achieved by Northern blotting. Total RNA was extracted from the following rat tissues:- postnatal day 1 (P1) and P10 brain, then P10 heart, lung, spleen, kidney, thymus, skeletal muscle, liver and testis. P1 is a time in development just before myelination by oligodendrocytes begins in the brain and the genes which are specifically involved are switched on. At P10 active myelination is occurring and such genes are highly expressed. Northern blotting of brain RNA from these two time points allowed the identification of mRNAs whose expression may be upregulated during this time indicating possible involvement in the process of myelination.

Total RNA (10 μg) from each of the different rat tissues were run out on a 0.8% formaldehyde agarose electrophoresis gel to separate the different RNA molecules according to size. Figure 3.1 shows a representative gel demonstrating approximately equal loadings for each of the tissues analysed. The large (28S) and

Table 3.1 List of known sequences isolated from the subtractive library.

α-2, 3-sialyltransferase	1
Albino rat cDNA for 16kDa subunit of vacuolar H(+) ATPase	1
Amyloid precursor-like protein mRNA	1
Apolipoprotein	1
α-tubulin	2
β-tubulin	1
CNPase	1
Cysteine protease	3
DAP kinase	1
Faeroesyltransferase beta subunit	1
Ferritin light chain gene	1
fyn proto-oncogene	1
Human breakpoint cluster region (BCR) gene	1
Human ras-like protein	3
Long chain acyl-CoA dehydrogenase	1
LLRep3 protein mRNAfrom a repetitive element	1
MAG	1
MBP	31
mitochondrial cytochrome c oxidase subunit	3
mRNA from Leydig cell hypercalcemic tumour	1
mRNA for major excreted protein (MEP)	1
Mycobacteriophage genome	1
NADH-ubiquinone oxidoreductase subunit mRNA (mitochondrial)	1
Phosphatidylethanolamine budding protein mRNA	1
PLP	1
Prostaglandin D-synthetase mRNA	1
Prostaglandin-H-2 D-isomerase	1
rah=ras-related homologue	1
rhoG mRNA for GTPase	1
SRP receptor	1
Sulfated glycoprotein	2
Suilisol mRNA	1
Talin mRNA	1
thaliaria cDNA	1
Thymosine beta-4 mRNA	1
Transcription factor	2
Transglutaminase mRNA	1
Triosephosphate isomerase mRNA	2
Tyrosine kinase	1
Ubiquinone oxidoreductase complex	1
TOTAL	80

Figure 3.1 A representative 0.8% formaldehyde agarose Northern gel before transfer demonstrating equal loadings of total RNA for each rat tissue analysed. The large (28S) and small (18S) ribosomal subunits indicate that the RNA has not degraded.


P1 brain
P10 brain
Heart
Lung
Kidney
Thymus
Muscle
Liver
Testis
9.5 kb
7.5 kb
4.4 kb
2.4 kb
1.4 kb
0.2 kb
28S
18S

small (18S) ribosomal subunits are clearly visible indicating that the RNA was intact.

Figure 3.2 shows six Northern blots with different cDNA probes. Probe OL0010 is clearly identifying an mRNA of approximately 4.4 kb which is present in all the tissues analysed. However, five of the 86 novel cDNA clones analysed hybridised only to RNA molecules present in brain and hence proved to be specific to the nervous system by not hybridising with mRNAs from any other tissue. These five clones were also hybridised with total RNA isolated from sciatic nerve of the peripheral nervous system (PNS), to deduce if any exhibited CNS specific expression. Optic nerve total RNA was analysed too. The optic nerve has been described as the simplest part of the CNS since there are no neuronal cell bodies present and no mRNAs in the nerve axons (Raff, 1989; Raff et al., 1987). Therefore it is reasonable to deduce that any signal detected from the hybridisation of clones with optic nerve total RNA indicates expression in glial cells.

Clone OL0003 identifies a very large mRNA molecule with a size in excess of 9.5 kb and which may be upregulated in brain from P1 to P10. Its presence in sciatic nerve indicates expression in the PNS and a signal in optic nerve indicates expression in CNS glial cells. Clone OL0587 identifies an mRNA between 7.5 kb and 9.5 kb which is present in sciatic and optic nerves and is therefore expressed by glial cells of both the peripheral and central nervous systems.

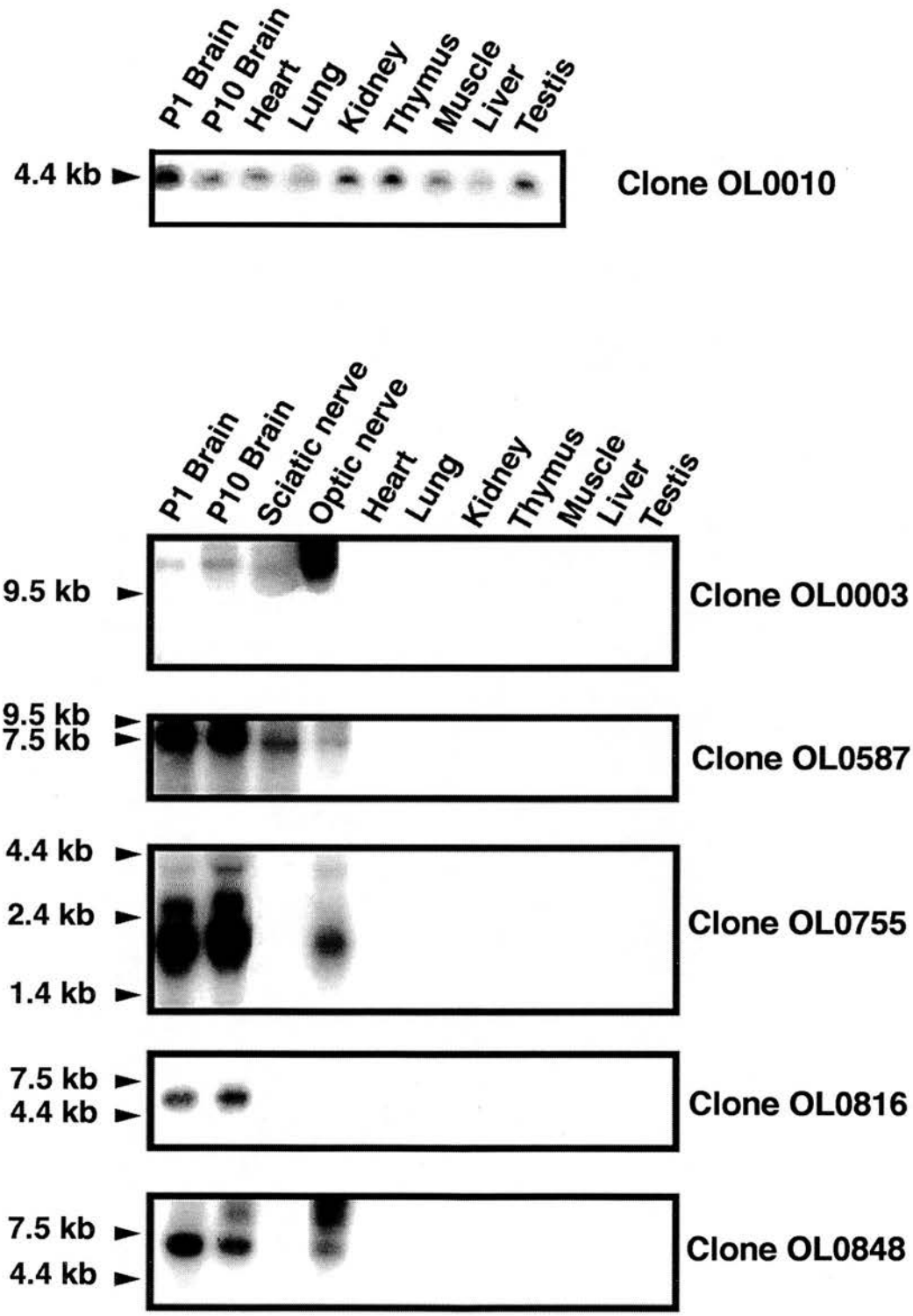
Clone OL0755, however, is expressed exclusively in the central nervous system; there is no indication of it in the sciatic nerve. The expression in optic nerve implicates its presence in CNS glial cells i.e. in oligodendrocytes and/or astrocytes. It is an interesting clone in the fact that it recognises more than a single mRNA molecule. At least two mRNAs of approximately 2 kb and 3 kb are identified by it. Possibly a third species of about 4 kb is also detected although this may be due to non-specific signal from the large ribosomal subunit.

Clones OL0816 and OL0848 both identify single mRNAs of between 4.4 kb and 7.5 kb and show a pattern of expression which is specific to the central nervous system.

Summary

From the Northern blot analysis it can be deduced that clone OL0003 is expressed by glial cells because of signal in optic and sciatic nerves. It may also be expressed by neurons due to signal in brain. Clone OL0587 is also expressed in glial cells but may also be expressed by neurons. Clone OL0755 is expressed by CNS glia but possibly also by neurons. This clone identifies two mRNAs which may be

Figure 3.2 Northern blots of total RNA from a variety of rat tissues, transferred to Hybond-N membrane and probed with 6 different cDNA probes which had been ^{32}P labelled by random priming. Clone OL0010 is clearly present in all tissues analysed. Clones OL0003, OL0587, OL0755, OL0816 and OL0848 are all clearly shown to be nervous system specific.


P1 Brain
P10 Brain
Heart
Lung
Kidney
Thymus
Muscle
Liver
Testis
4.4 kb
Clone OL0010
P1 Brain
P10 Brain
Sciatic nerve
Optic nerve
Heart
Lung
Kidney
Thymus
Muscle
Liver
Testis
9.5 kb
Clone OL0003
9.5 kb
7.5 kb
Clone OL0587
4.4 kb
2.4 kb
1.4 kb
Clone OL0755
7.5 kb
4.4 kb
Clone OL0816
7.5 kb
4.4 kb
Clone OL0848

related by alternative splicing mechanisms. Clone OL0816 must only be expressed by neurons since there was no signal in optic or sciatic nerves. Clone OL0848 is expressed by CNS glia but possibly also by neurons.

3.2 Expression of the novel nervous system specific cDNA clones in cultured glial cells as deduced by ISH studies

Having established that 5 of the clones have an expression pattern specific to the nervous system, investigations were carried out to deduce if any of these clones were expressed by specific cell types. From the Northern blot data, the signals in optic nerve and/or sciatic nerve already suggest that all the clones, except OL0816, are expressed by glial cells. Expression of clone OL0816 specifically in brain and not in either of the two nerves, implicates expression by neurons. In situ hybridisation (ISH) studies on cultured glial cells were used to investigate this question.

The five clones have different sized inserts as clearly shown in Figure 3.3. The approximate sizes of the inserts for clones OL0003, OL0587, OL0755, OL0816 and OL0848 are 1.2 kb, 2.4 kb, 1.1 kb, 0.8 kb and 1.1 kb respectively. Riboprobes were prepared from the purified plasmid DNA for each clone. As an indication of the success of transcription the amount of incorporated radioactivity into the newly synthesised riboprobe was calculated. Table 3.2 shows representative radioactive readings for the five riboprobes that were made and indicate high incorporation of radiolabel. From these figures the volume of 0.01 M DTT in which to resuspend the probe to give a concentration of 1 ng/µl/kb could be calculated, as described in the Materials and Methods.

Figures 3.4 and 3.5 show the results of the ISH experiments done on the cultured glial cells. The antibody against the O4 surface antigen is clearly labelling cells of the oligodendrocyte lineage. These cells show the characteristic morphology of many highly branched processes extending and radiating from the cell body. In some cases the early stages of myelin sheet production is apparent at the ends of processes.

Astrocytes present in the cultures have also been clearly defined with the anti-GFAP monoclonal antibody GA5, although it is not able to distinguish between type 1 and type 2 astrocytes. A typical astrocytic morphology is apparent in these cells with much longer, less branched and fewer processes radiating from their cell bodies than observed in oligodendrocytes. It is also possible to see filamentous staining in some of these cells.

In figure 3.4 it can be seen that clone OL0003 is expressed by $O4^+$ oligodendrocytes by the clustering of silvergrains around the cell body. The silver grains are indicative of radioactive antisense riboprobe that has hybridised with native mRNA molecules present in the cell. This clone does not, however, appear to

Figure 3.3 Restriction digests of plasmid DNAs for the 5 brain specific clones of interest. The purified plasmid DNAs were digested with the restriction enzymes NotI and SalI to release the inserts from the pSPORT1 vector and then run out on a 1% agarose electrophoresis gel. pSPORT1 runs as a band at 4 kb. The 5 clones clearly have different inserts with differing sizes.

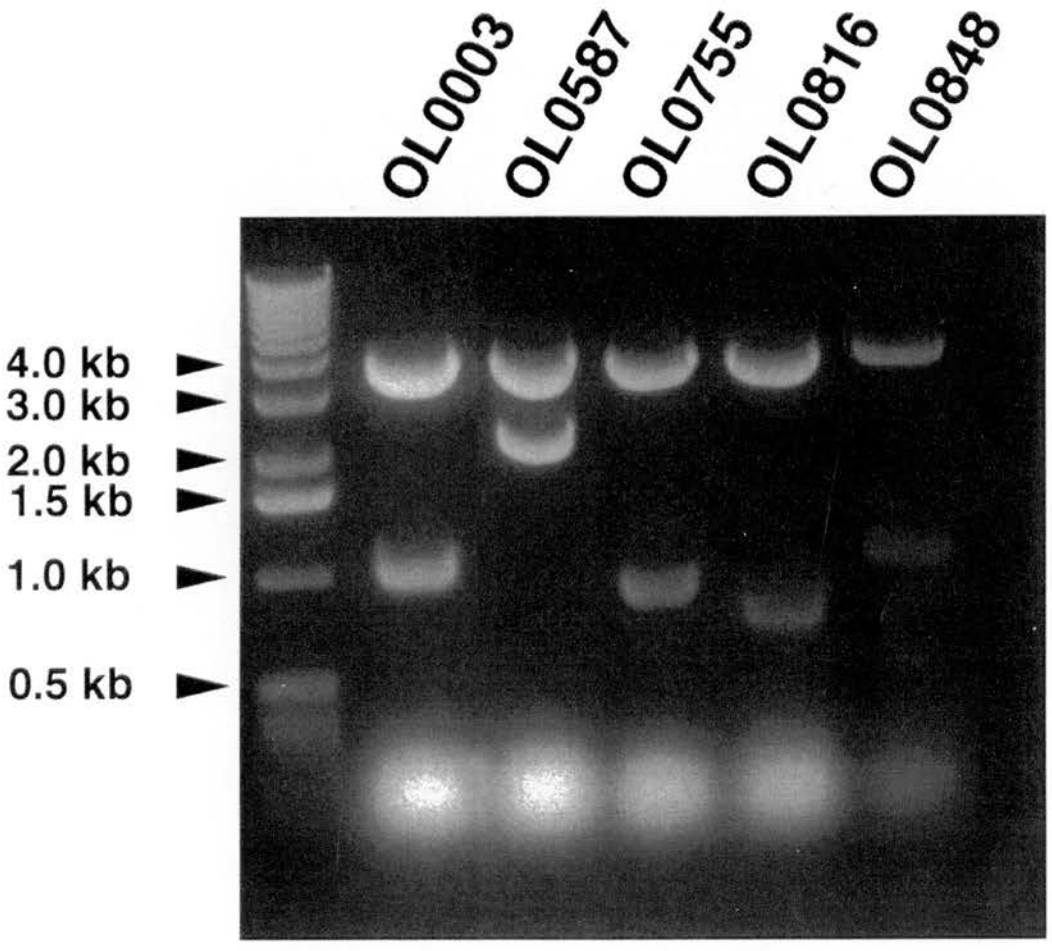

OL0003
OL0587
OL0755
OL0816
OL0848
4.0 kb
3.0 kb
2.0 kb
1.5 kb
1.0 kb
0.5 kb

Table 3.2 Representative radioactive readings for the 5 riboprobes synthesised. From the measured counts per minute (CPM) of precipitated RNA solution (Total), resuspended pelleted RNA (Pellet) and the supernatant (Supernatant) the incorporation of [^{35}S]-UTP during transcription was calculated. As can be seen from the table, incorporation was high.

Clone	CPM Total	CPM Pellet	CPM Supernatant	% Unincorporated	% Incorporated
003 AS	414497	103283	51845	12.5	87.5
003 S	743234	175273	73969	10.0	90
587 AS	412923	173834	34887	8.4	91.6
587 S	713069	377761	52623	7.4	92.6
755 AS	405312	203875	61076	15.0	85
755 S	411120	172459	31915	7.7	92.3
816 AS	539615	131253	23478	4.35	95.65
816 S	785840	931132	61824	7.9	92.1
848 AS	265320	126993	71311	26.9	73.1
848 S	379457	359931	111254	29.3	70.7

Figure 3.4 Double label immunocytochemistry and ISH analysis of glial cells in culture. ^{35}S-labelled riboprobes prepared from each cDNA were used to detect mRNA in cultures of glial progenitor cells which had been allowed to differentiate for four days. Cells of the oligodendrocyte lineage are fluorescently labelled with the monoclonal antibody 04 coupled to TRITC (red colour) and astrocytes are labelled by a monoclonal antibody against GFAP (GA5) which was also coupled to TRITC. Clones OL0003, OL0587 and OL0848 show expression specific to oligodendrocytes by a clustering of silver grains over their cell bodies (yellow/green dots); none show expression in astrocytes. (OL003=OL0003, OL587=OL0587, OL848=OL0848).

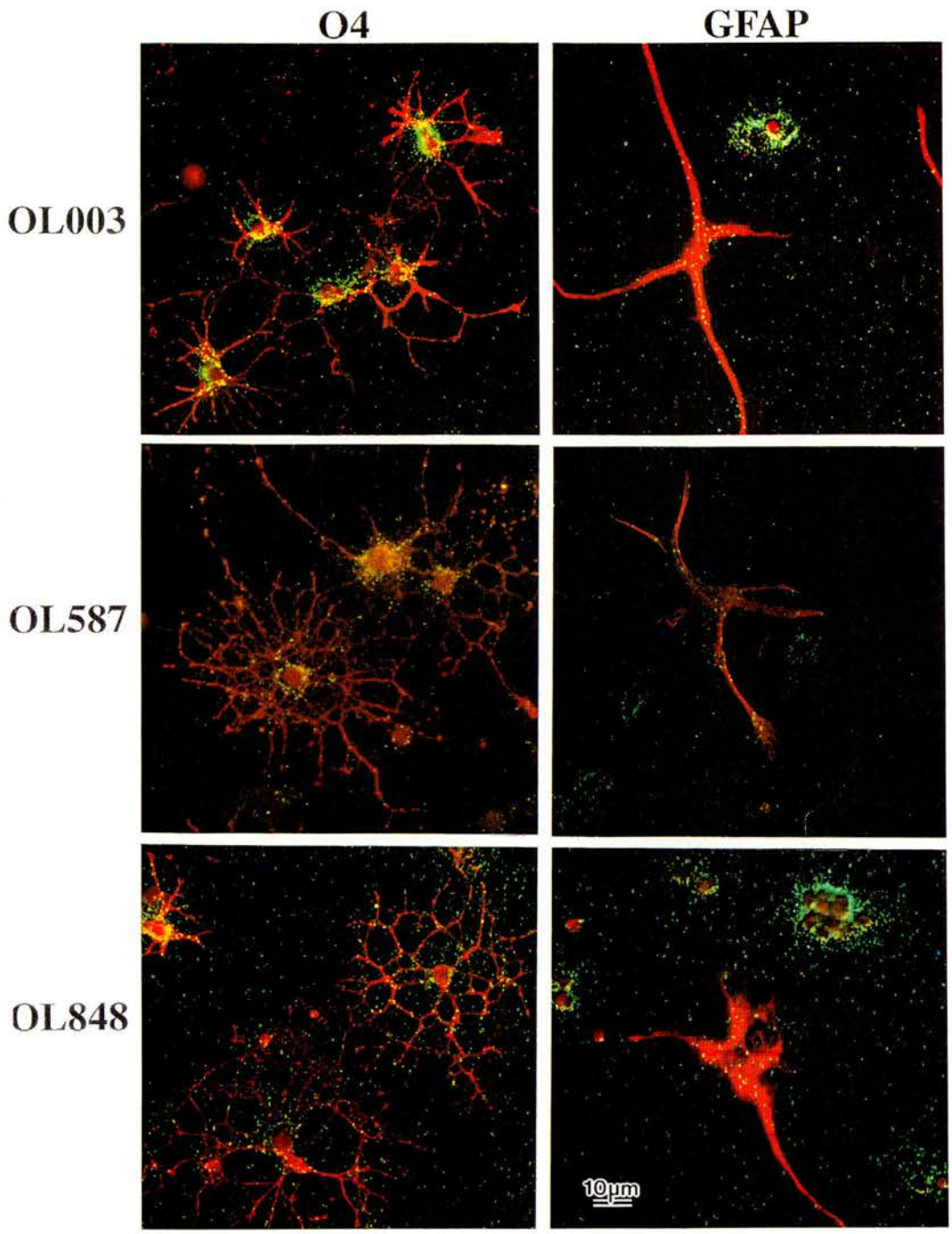
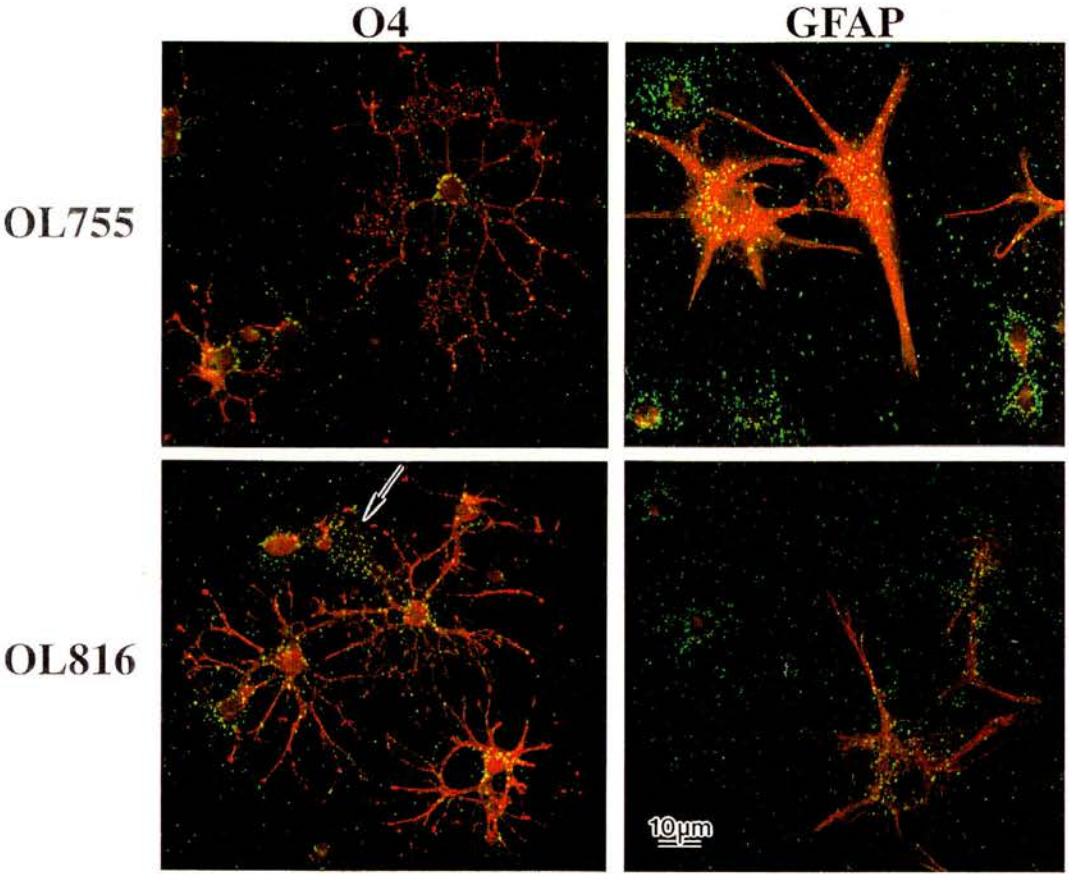

O4
GFAP
OL003
OL587
OL848
10μm

Figure 3.5 Double label immunocytochemistry and ISH analysis of glial cells in culture. ^{35}S-labelled riboprobes prepared for each cDNA were used to detect mRNA in cultures of glial progenitor cells which had been allowed to differentiate for four days. Cells of the oligodendrocyte lineage are fluorescently labelled with the monoclonal antibody 04 coupled to TRITC (red colour) and an anti-GFAP monoclonal antibody (GA5) coupled to TRITC labels astrocytes. Clones OL0755 and OL0816 appear to be expressed by both oligodendrocytes and astrocytes. Clone OL0816 is also labelling a cell which is not fluorescently labelled with 04 (indicated by an arrow); it may be an astrocyte, fibroblast or some other cell type. (OL755=OL0755, OL816=OL0816).


O4
GFAP
OL755
OL816
10μm

label GFAP+ astrocytes. Clones OL0587 and OL0848 also appear to be expressed only by oligodendrocytes and not by astrocytes.

Figure 3.5 shows that clones OL0755 and OL0816 are expressed by both oligodendrocytes and astrocytes in culture although clone OL0816 is very much more weakly expressed. There are clear clusterings of silver grains over the cell bodies of both cell types when using these 2 riboprobes.

Conclusions

Clone OL0003 is expressed by cultured oligodendrocytes but not by astrocytes. Combined with the results from the Northern blot this indicates expression by myelinating CNS glia (oligodendrocytes) and possibly also neurons. Clone OL0587 is also expressed by oligodendrocytes in culture but not astrocytes. Therefore results so far indicate expression by oligodendrocytes and possibly also neurons. This is also the case for clone OL0848.

Clone OL0755 is expressed by both oligodendrocytes and astrocytes in culture. Therefore results so far indicate expression by glia and possibly also by neurons. Clone OL0816 is also expressed by oligodendrocytes and astrocytes in culture, however this result does not agree with the Northern blot which implicated expression only by neurons. This conflict in results may indicate a difference in sensitivity between the two techniques or a difference in the expression in vitro (cultured cells) from in vivo (dissected nerves).

3.3. Isolation of a full length cDNA for clone OL0755

At this stage of the work it was decided to focus on a single clone. Clone OL0755 was chosen for two main reasons. First, it is an interesting clone in that it is expressed by both oligodendrocytes and astrocytes in culture as seen by ISH. Secondly, the pattern of expression as deduced by Northern blotting is unique in that it is the only one of the five brain specific clones to identify more than a single mRNA molecule. Therefore for these reasons clone OL0755 was chosen for further investigation.

Clone OL0755 has an insert which is approximately 1 kb and yet it identifies mRNA molecules on Northern blots which are approximately 2 kb and 3 kb. There is a possible third species identified at about 4 kb, although this may be due to background signal from the large ribosomal subunit. Clone OL0755 therefore cannot be a full length cDNA since the mRNA molecules it identifies on Northern blots are clearly greater in size.

To obtain a full length cDNA, clone OL0755 was used to probe a cDNA library. The cDNA library analysed was an oligo dT primed, directionally cloned (NotI -> SalI), mixed age (1 day, 15 day and 16 weeks) rat brain library in λgt22A. A library constructed from rat brains of these ages was considered a good source of related clones since clone OL0755 identifies mRNAs expressed at P1 and P10 in rat brain on Northern blots. Partial sequencing of clone OL0755 shows that the extreme 3′ end is complete since it includes a polyadenylation signal and a polyA tail. Therefore an oligo dT primed library is a good source of larger cDNAs with additional 5′ sequence and complete 3′ sequence.

In the first round of hybridisation screening the entire cDNA library was plated on four plates each with 60,000 clones. Filter lifts were taken which were then all hybridised with clone OL0755 which had been radioactively labelled with [α-^{32}P] dCTP by random priming. Autoradiography of the hybridised filters allowed identification of the plaques which contained cDNA clones which had hybridised with clone OL0755 and hence appeared to have the same DNA sequence. From the autoradiographs 28 positive plaques were identified and 25 plugs containing positive plaques were isolated from the plates.

The resultant plug lysates were analysed by PCR as an additional test for the presence of clone OL0755 sequence. Primers 11F and 7R were used to amplify a 342 bp fragment found at the 5′ end of clone OL0755 (see Materials and Methods for details). These primers were chosen from the partial sequence obtained for clone OL0755. Of the 25 plug lysates from the primary hybridisation screen only 15

amplified a band of approximately 300 bp during PCR with these primers as can be seen in Figure 3.6A. Therefore only 15 out of the 25 plug lysates definitely contain cDNA clones with the same sequence as clone OL0755. The other lysates may contain cDNA clones which are smaller. Since the library was oligo dT primed the extreme 5′ end sequence of clone OL0755 may not be present in these clones and therefore the PCR amplification with primers 11F and 7R would not be possible. Alternatively the lysates may not contain cDNA clones with the same sequence as OL0755.

In an attempt to estimate the amount of additional 5′ sequence present in clones related to OL0755, a PCR amplification was done with these plug lysates using primer 601, a forward primer which is present in the λgt22A vector sequence, and primer 7R. Not all the 15 plug lysates amplified. The largest DNA fragment amplified was approximately 500-600 bp indicating an extra 200-300 bp of 5′ sequence. It is possible, however, that some clones did not amplify because conditions were unsuitable. Figure 3.6B shows the amplification of potential additional 5′ sequence.

Six plug lysates, 2, 3, 12, 13, 19 and 22, were chosen for the next round of screening. 200 plaque forming units of each were plated out and the filter lifts from each plate were hybridised this time with digoxigenin labelled clone OL0755. Positive plaques were identified following a colour reaction. Of the six plates in the second round of screening plate 12 had no positive plaques, plate 2 had three positives, plate 22 had two positives, and plates 3, 13, and 19 had only one each. Plug lysates from the eight positive plaques were amplified by PCR with primers 11F and 7R to confirm the presence of clone OL0755 sequence. Figure 3.7 shows this amplification. Only the plug lysates from plates 3, 19 and one from 22 amplified.

High titre lysates were prepared from these three and the phage DNA isolated from each. Digestion of the purified DNA with the restriction enzymes NotI and SalI led to estimation of the size of the inserts and therefore whether any were possible full length cDNAs for clone OL0755. Figure 3.8 shows the digests of the phage DNA for the three clones. From this point these 3 clones will be called 3, 19 and 22 respectively. The insert of clone 3 is about 2 kb, that of clone 19 is about 1.5 kb and clone 22 has one of approximately 3 kb. These insert DNA fragments were purified and ligated into the vector pSPORT1 and then sequenced.

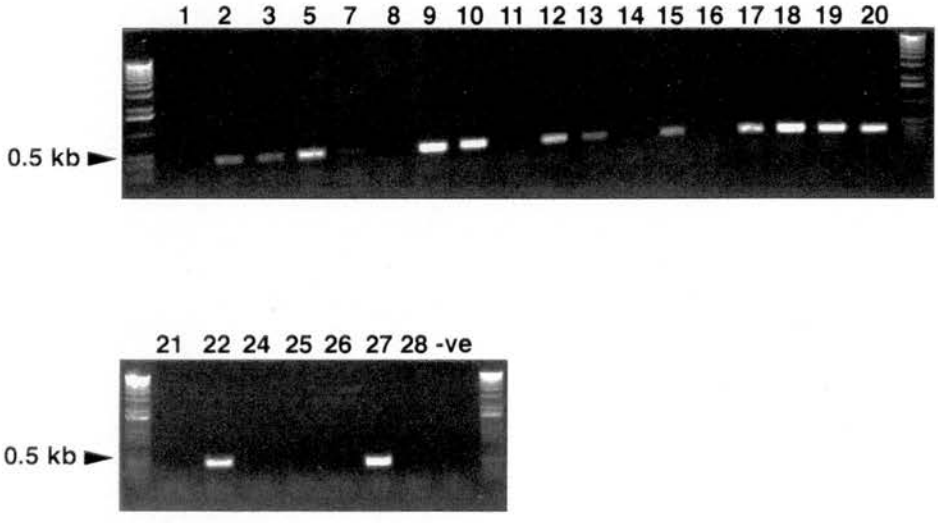
The clones were sequenced at both ends using the T7 and M13F primers. Clone 19 was discarded as an artefact since its sequence did not match clone OL0755 and other clone OL0755 primers produced no sequence at all. Clone 3

Figure 3.6 PCR amplification of plug lysates from the primary hybridisation screen of the cDNA library.

A shows the amplification with primers 11F and 7R to confirm the presence of clone OL0755 sequence. 15 of the 25 plug lysates have amplified clone OL0755 sequence. The negative control which had no template DNA added is clear.

B shows the amplification of those plug lysates positive for clone OL0755 sequence with primers 601 and 7R to estimate the amount of additional 5´ sequence. Not all lysates have amplified, possibly because conditions were not optimal. Again the negative control is clear.

A. Primers 11F and 7R



B. Primers 601 and 7R

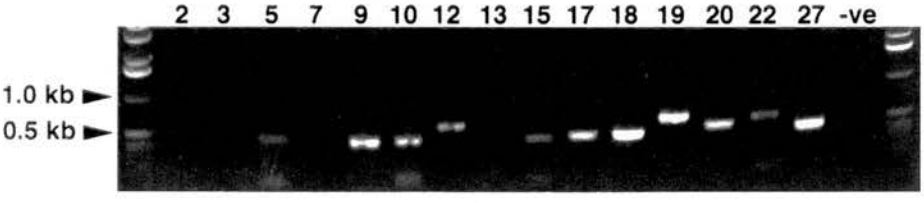


Figure 3.7 PCR amplification of the plug lysates from the secondary hybridisation screen of the cDNA library. Amplification was done with primers 11F and 7R to confirm the presence of clone OL0755 sequence. Only plug lysates 3, 19 and 22a amplified clone OL0755 sequence.

Primers 11F and 7R

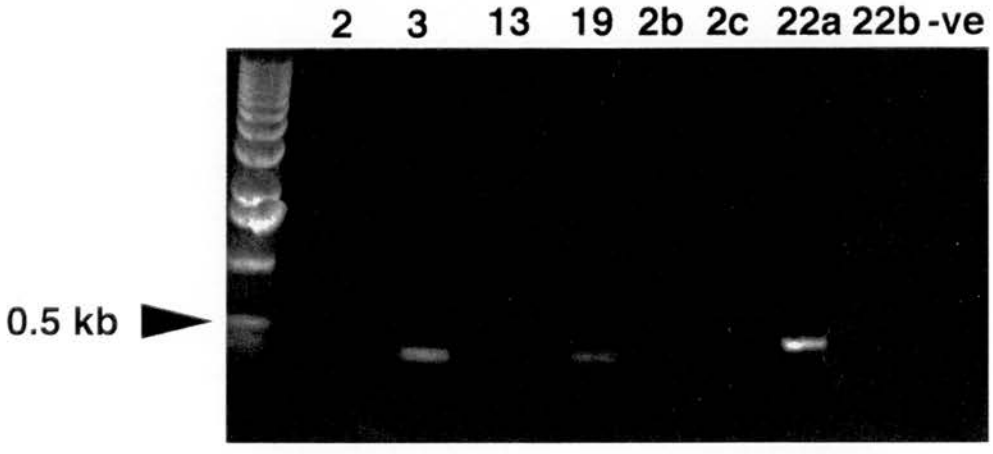
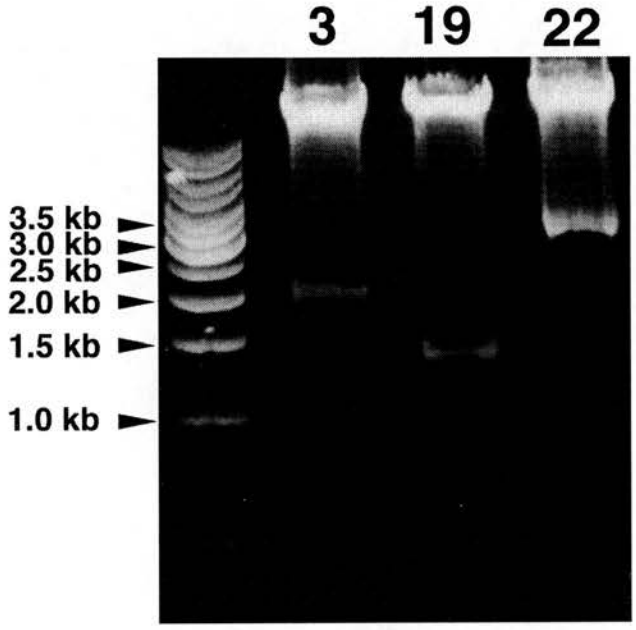


Figure 3.8 Digests of purified λphage DNA with the restriction enzymes NotI and SalI to release insert DNA fragments. The uppermost bands on the gel are the digested λgt22A phage DNA. Clone 3 has an insert of about 2 kb, clone 19 an insert of about 1.4 kb and clone 22 an insert of about 3 kb.


3
19
22
3.5 kb
3.0 kb
2.5 kb
2.0 kb
1.5 kb
1.0 kb

produced unreadable sequence and so was also discarded as an artefact. Clone 22, however produced sequence that matched clone OL0755 at the 3′ end, including a polyadenylation signal and polyA tail, and clear readable sequence at the 5′ end. Therefore it appeared that this 3 kb clone 22 could be a full-length cDNA for clone OL0755 since it agrees in size with the 3 kb mRNA identified on the Northern blot. It was named OL0755-A.

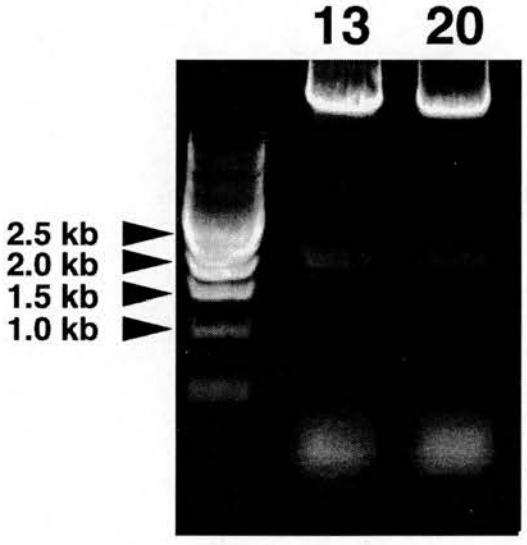
Since a smaller mRNA molecule of about 2 kb was also identified on the Northern blot it was decided to repeat the secondary hybridisation screen in the hope of isolating a second potential full length cDNA. The plug lysates 2, 3, 13 and 20 from the primary screen were chosen for a second round of screening. These lysates all amplified a band of about 300 bp with primers 11F and 7R indicating the presence of clone OL0755 sequence. Although lysates 2 and 3 had already undergone a secondary screen yielding no full length clones they were chosen again to see if sensitivity of the screen was improved by using radioactively labelled probe instead of digoxigenin labelled probe.

The secondary hybridisation screen using radioactively labelled clone OL0755 as the probe identified at least one positive plaque on each of the four plates. Plate 2 had six positives, plate 3 had three, plate 13 had one and plate 20 had six positive plaques. The plug lysates from the plaques with the strongest signal on each plate were taken through a third hybridisation screen to ensure purification of the plaques of interest. High titre lysates were prepared from a positive plaque on each plate and the phage DNA purified from each. Following digestion with the restriction enzymes NotI and SalI to release the inserts, only lysates from plates 13 and 20 appeared to contain cDNA inserts of 2-2.5 kb as shown in Figure 3.9. From now on they will be referred to as clones 13 and 20.

The purified insert DNA fragments were ligated into PGEM11zf and sequenced. When sequenced with the primers T7 and M13F clone 20 produced sequence which did not relate to clone OL0755 or clone OL0755-A and so was discarded as an artefact. Clone 13 did produce related sequence to clone OL0755 and clone OL0755-A agreeing exactly at the 3′ end and possessing a polyadenylation signal and polyA tail. The 5′ end gave clear readable sequence which matched exactly with clone OL0755-A 5′ sequence. It therefore appeared that clone 13 was probably the full length cDNA of clone OL0755 encoding the 2 kb mRNA molecule detected on the Northern blot and was therefore named clone OL0755-B.

Clones OL0755, OL0755-A and OL0755-B were all fully sequenced from both directions giving the nucleotide compositions for both DNA strands. Clone

Figure 3.9 Digests of purified λphage DNA with the restriction enzymes NotI and SalI to release insert DNA fragments. The uppermost bands on the gel are the digested λgt22A phage DNA. Both clones 13 and 20 have inserts of about 2 kb.


13
20
2.5 kb
2.0 kb
1.5 kb
1.0 kb

OL0755-A is 2771bp in size and clone OL0755-B is 2050bp. Both these sizes compare well with the mRNAs identified by Northern blotting and thus support the view that these clones are full length. Both clones have a polyadenylation signal (AATAAA) followed by a poly A tail 13 bp downstream which confirms that the 3′ ends of the clones are complete.

Figures 3.10 and 3.11 show the full nucleotide sequences for each clone. Sequence analysis predicts that clone OL0755-A has 127 bp of untranslated 5′ sequence followed by a 1605 nucleotide open reading frame (ORF) encoding a protein of 535 amino acids. Clone OL0755-B has 247 bp of 5′ non-coding sequence followed by an ORF of 1311 nucleotides encoding a protein of 437 amino acids. Both clones have a common start site for translation. The initiation codon is preceded by at least one in frame stop codon 15 bp upstream suggesting that this is the correct initiation site. Comparison with the Kozak consensus sequence for the start of translation (GCCGCC(A/G)CCATGG) (Kozak, 1987; Kozak, 1989) however, does not show great similarity (clones OL0755-A and OL0755-B:-ACATCTGGATGG), but of the two positions postulated to be of particular importance, -3 and +4, one is in agreement (G at position +4). Clones OL0755-A and OL0755-B are related to each other at the nucleotide level in a manner suggestive of possible alternative splicing of a common gene. High similarity at the amino acid level also supports this likelihood. Figure 3.12 shows schematically how these clones are related to one another and to clone OL0755.

Analysis of the translated products for each clone was done using MacVector software. Clone OL0755-A has a predicted MW of 59690 and a pI of 6.59. A hydrophilicity profile (Kyte and Doolittle, 1982) (see Figure 3.13) was done with constants averaged over 7 amino acids and showed the protein to be mostly hydrophilic in nature except for a significantly hydrophobic 13 amino acid stretch from Ser-457 to Gln-469 which has predicted beta-sheet secondary structure An extended beta strand requires only 9 residues to span a lipid bilayer (Gardinier et al., 1992) and so this region may be a potential transmembrane sequence. Clone OL0755-A also has one predicted ATP/GTP binding motif (GEVLDGKT) at amino acid 263, a TGF binding motif (CAPAGPVSWF) at amino acid 118 and 7 possible N-glycosylation sites at positions 159, 191, 441, 492, 527, 532 and 540.

Clone OL0755-B has a predicted MW of 49252 and a pI of 5.23. It, like clone OL0755-A, is mostly hydrophilic in nature except for one potential membrane spanning region at Thr-168 to Glu-188 which has possible α-helix structure. Figure 3.13 shows the plot. One ATP/GTP binding motif was identified at amino acid 270, a TGF binding motif at amino acid 158 and two N-glycosylation sites were

Figure 3.10 The complete nucleotide and deduced amino acid sequence of clone OL0755-A. The protein has 535 amino acids (including the initiation methionine) and a calculated molecular weight of 59,690. The sequence in bold type (AATAAA) indicates the polyadenylation signal. The underlined amino acid sequence denotes a 23 amino acid peptide used to raise a polyclonal antibody against. Primers 16F and 21R used to generate clone OL0755-A(S) by PCR amplification are also indicated.

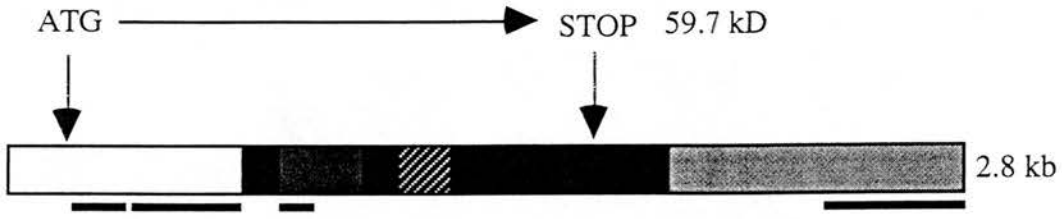
```
GCTCGGAGCAGTCCCTCCCCGGCCTTGTTTTGCTCCGGAATCGCTGCTGGGGGAGGGAGCGAAGGGGCCGGCCCT  75

AGGTGTCCAGGCCGGGGGCAGCGGGCGGCGGCAGTAGTGGCCGCACATCTGGATGGAAGCGAGCTTTGTCCAGAC  150
                                                    M  E  A  S  F  V  Q  T    8

CACCATGGCTCTGGGGCTGCCCTCCAAGAAAGCATCTTCCCGCAACGTGATCGTGGAGCGCAGGAACCTGATCAC  225
T  M  A  L  G  L  P  S  K  K  A  S  S  R  N  V  I  V  E  R  R  N  L  I  T   33

CGTGTGCAGGTTCTCTGTGAAAACCTTGCTAGAGAAGTACACAGCAGAACCCATCGATGATTCATCCGAGGAGTT  300
 V  C  R  F  S  V  K  T  L  L  E  K  Y  T  A  E  P  I  D  D  S  S  E  E  F   58

TGTTAACTTCGCAGCCATTTTAGAGCAGATCCTCAGCCACCGATTTAAAGCTTGTGCCCCAGCAGGTCCAGTGAG  375
 V  N  F  A  A  I  L  E  Q  I  L  S  H  R  F  K  A  C  A  P  A  G  P  V  S   83

CTGGTTCAGCTCAGATGGGCAACGGGGCTTCTGGGACTATATCCGGCTGGCCTGCAGCAAAGTGCCCAACAACTG  450
 W  F  S  S  D  G  Q  R  G  F  W  D  Y  I  R  L  A  C  S  K  V  P  N  N  C   108

CGTAAGCAGCATCGAGAACATGGAGAACATCAGCACAGCTCGAGCCAAGGGCCGGGCGTGGATCCGGGTGGCTCT  525
 V  S  S  I  E  N  M  E  N  I  S  T  A  R  A  K  G  R  A  W  I  R  V  A  L   133

GATGGAGAAGCGTATGTCAGAATACATCACTACAGCTCTTCGGGACAACCGAACTACCAGACGGTTCTATGACTC  600
 M  E  K  R  M  S  E  Y  I  T  T  A  L  R  D  N  R  T  T  R  R  F  Y  D  S   158

CGGAGCCATCATGCTGCGAGAGGAAGCCACTGTCCTCACAGGGATGCTGATCGGACTCAGCGCTATCGACTTCAG  675
 G  A  I  M  L  R  E  E  A  T  V  L  T  G  M  L  I  G  L  S  A  I  D  F  R   183

GTGGGGTCTGCTTGGCGGTGGTGAAGGGGACTTTCTCACCCACAACTGTCCATCGCCCCCTCATTCCCTCCCCCC  750
 W  G  L  L  G  G  G  E  G  D  F  L  T  H  N  C  P  S  P  P  H  S  L  P  P   208

ACCTCTGTGGCTCTTCTGCCCCAGCTTCTGTCTAAAGGGCGAAGTTCTGGACGGGAAGACGCCGGTGGTCATCGA  825
 P  L  W  L  F  C  P  S  F  C  L  K  G  E  V  L  D  G  K  T  P  V  V  I  D   233

TTACACACCCTACCTAAAATTCACCCAAAGCTACGACTACCTGACGGATGAGGAGGAGAGGCACAGTGCAGAGAG  900
 Y  T  P  Y  L  K  F  T  Q  S  Y  D  Y  L  T  D  E  E  E  R  H  S  A  E  S   258

CAGCACCAGCGAGGACAACTCACCAGAACACCCCTACCTGCCTCTCGTCACCGATGAAGACAGCTGGTACAACAA  975
 S  T  S  E  D  N  S  P  E  H  P  Y  L  P  L  V  T  D  E  D  S  W  Y  N  K   283

GTGGCACAAGACGGAACAGAAGTTTCGCATTGTCTACGCTCAGAAGGTGCGCGGGGCGAGGCGCGGGGAGCTGGG  1050
 W  H  K  T  E  Q  K  F  R  I  V  Y  A  Q  K  V  R  G  A  R  R  G  E  L  G   308

CGCTGTGGGGGCAGGGCCTGGGCTGGGACCCCAGCCGGGACCCCTTTGGGTGTCCGGGCTGAGCCGGCGCCCCGC  1125
 A  V  G  A  G  P  G  L  G  P  Q  P  G  P  L  W  V  S  G  L  S  R  R  P  A   333

AGGATACCTGGAGGAGCTGGTGCGTCTGCGGGAGTCGCAGCTGAAGGACCTGGAGGCGGAGAACCGGCGGCTGCA  1200
 G  Y  L  E  E  L  V  R  L  R  E  S  Q  L  K  D  L  E  A  E  N  R  R  L  Q   358

GCTGCAGCTGGAGGAGGCAGCGGCACAAAATCAGCGTGAGAAGCGGGAGCTGGAAGGAGTGATCCTGGAGCTGCA  1275
 L  Q  L  E  E  A  A  A  Q  N  Q  R  E  K  R  E  L  E  G  V  I  L  E  L  Q   383
                                                                  16F->
GGAGCAGCTGGTGAGCCCGCCTCCCGCCCTGACCCCTGTGAACCCCAACATGACTCCATTGGATGGGATCATGAG  1350
 E  Q  L  V  S  P  P  P  A  L  T  P  V  N  P  N  M  T  P  L  D  G  I  M  R   408

GACATCCAATTCGCATCAGCCCACTCATCCCATCCCCCTTTCCCAAGATCCTGTGGTGGCCAAACATTACCTGCC  1425
 T  S  N  S  H  Q  P  T  H  P  I  P  L  S  Q  D  P  V  V  A  K  H  Y  L  P   433

CCCCACCTTAAAACCTGGCAGCTTCCAATACCCATCAGCTAGCGACACCAACCATTCCTCACAGGACCTAAGCAG  1500
 P  T  L  K  P  G  S  F  Q  Y  P  S  A  S  D  T  N  H  S  S  Q  D  L  S  R   458

GCTTGTTTTATTGTGCACCATCCCCATTGTACAGACCTCTGATAGGCATCACCCAGTGACACCCCCTCCCCCAAC  1575
 L  V  L  L  C  T  I  P  I  V  Q  T  S  D  R  H  H  P  V  T  P  P  P  P  T   483

CTGGAACATTACCTCCCCGAACCTGACCCAGCTAGCTAGCACAAACCCCACCCCTTCAAGAACACCCACCCTTTC  1650
 W  N  I  T  S  P  N  L  T  Q  L  A  S  T  N  P  T  P  S  R  T  P  T  L  S   508

TACTCTCCTCCCGACAACATCCCAACTGATTTCTAAACACTCCATCACTAATCCTGGCCACCTTACTAAGGAGCA  1725
 T  L  L  P  T  T  S  Q  L  I  S  K  H  S  I  T  N  P  G  H  L  T  K  E  Q   533

GAACTTGTGAGCCTCTTACCTCAGAGATCCTAGTGCCATAATTCCAGACATGTGCCACCCTCCCTGAGCAGCATA  1800
 N  L                                                                        558
                                                                      <-21R
TATCCCCATTTTTTTTACCACTCAAATTTCCTTTCTAAACCCTGGCCCCCACCTTTGCTGAGACTTGCTTCCCTC  1875

TCCCAGCCAGGCCTGATCCCCCTCCCCCTCCCAGGACAGGTCTGATCCCCGGTGACCATGCCCCCCTGGCCCAGG  1950

GTTCCAAGGAGCTCACCACAGCCCTGGTCAACCAGTGGCCCTCCCTGAGCACACTCAGTAGACCGGAGGGTGCCA  2025

GCAACTCCAAGCTATTTCGGAGACACAGCTTCATGAGTACGGAGCCCCTGTCTGCAGAGGCCAGCCTGAGCTCAG  2100

ACTCCCAGCGCCTGGGTGAGGGCAAGAGGGACGAGGAACCCTGGGGCCCCATCGGGAAGGACCCCACGCCCTCCA  2175

TGCTGGGCCTCTGCGGCTCCCTGGCCTCCATCCCCAGCCTGCAAGTCCCTGGCGAGCTTCAAATCCAACGAATGC  2250

CTGGTGAGCGACAGCCCTGAGGGCAGCCCAGCACTCAGCCCCAGCTGAGGAGCAGCAAGGGCAATGCCAGCCCCA  2325

CCTGCCAGGGGCCATGGACAACTGCCACCTTTTGAGTCCCCCACCCCAGGCCACCCTTCAAGAACAATACCCAGC  2400

TAGCCTCAGGTCCCAGCTAGCCTCGGGTCTCTAGGGAAAGACAACCTGAGCCTCTTTCCTGCTTCAGCTTCCTGC  2475

CAAAGAGGCAGGAGCTACATGGGGAAGTGGTGGGGCCAGGAGGGACGCCCAGGTTACAGGTCGTCAGTCCCCTGG  2550

GGAAGCCACTCCATTCCGTGGTCCTGAAGGCTGCCTGGTTCCTTCTGTTCATCTTCCACACTTGCCTCAGAAGCA  2625

GGTGGTCCAGCCCTGGCATTCCTGCTGCCCTGCCTCTGGTCTAACCCTGTGTACCCTCTGAAGTCACCCTTCCTC  2700

GGTACCTATGTGGGGAGAGATTAGGCA**AATAAA**AACCAGAGGACTGAAAAAAAAAAAAAAAAAAAAAAAAA  2775
```

Figure 3.11 The complete nucleotide and deduced amino acid sequence of clone OL0755-B. The protein has 437 amino acids (including the initiation methionine) and a calculated molecular weight of 49,252. The sequence in bold type (AATAAA) indicates the polyadenylation signal. The underlined amino acid sequence denotes a 23 amino acid peptide used to raise a polyclonal antibody against.

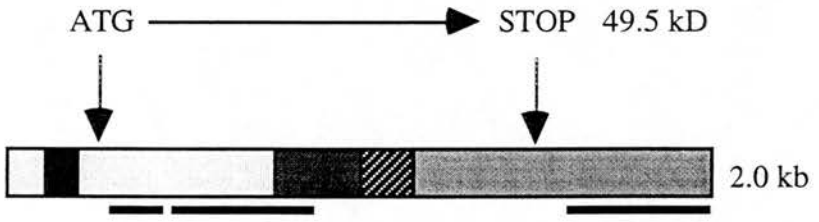
```
GGTGACGAGCCCCGGCTCAGCACCTCGGAGAGCTCCCTCCCCGGCCTTGTTTTGCTCCGGAATCGCTGCTGGGGG  75

AGGGAGCGAAGGGGCCGGCCCTAGGTGTCCAGGCCGAGGGATGGCAGTGAAGAGGACGAAGGGCCCCCCGGGGGG 150

CAGAGGGCCCAGGGCGTGATCCTGGCGAGGGGGGCCCGGGGGCCGCCCGGGTGGGGTGGGGGGCAGCGGGCGGCG 225

GCAGTAGTGGCCGCACATCTGGATGGAAGCGAGCTTTGTCCAGACCACCATGGCTCTGGGGCTGCCCTCCAAGAA 300
                    M  E  A  S  F  V  Q  T  T  M  A  L  G  L  P  S  K  K      18

AGCATCTTCCCGCAACGTGATCGTGGAGCGCAGGAACCTGATCACCGTGTGCAGGTTCTCTGTGAAAACCTTGCT 375
 A  S  S  R  N  V  I  V  E  R  R  N  L  I  T  V  C  R  F  S  V  K  T  L  L     43

AGAGAAGTACACAGCAGAACCCATCGATGATTCATCCGAGGAGTTTGTTAACTTCGCAGCCATTTTAGAGCAGAT 450
 E  K  Y  T  A  E  P  I  D  D  S  S  E  E  F  V  N  F  A  A  I  L  E  Q  I     68

CCTCAGCCACCGATTTAAAGCTTGTGCCCCAGCAGGTCCAGTGAGCTGGTTCAGCTCAGATGGGCAACGGGGCTT 525
 L  S  H  R  F  K  A  C  A  P  A  G  P  V  S  W  F  S  S  D  G  Q  R  G  F     93

CTGGGACTATATCCGGCTGGCCTGCAGCAAAGTGCCCAACAACTGCGTAAGCAGCATCGAGAACATGGAGAACAT 600
 W  D  Y  I  R  L  A  C  S  K  V  P  N  N  C  V  S  S  I  E  N  M  E  N  I    118

CAGCACAGCTCGAGCCAAGGGCCGGGCGTGGATCCGGGTGGCTCTGATGGAGAAGCGTATGTCAGAATACATCAC 675
 S  T  A  R  A  K  G  R  A  W  I  R  V  A  L  M  E  K  R  M  S  E  Y  I  T    143

TACAGCTCTTCGGGACAACCGAACTACCAGACGGTTCTATGACTCCGGAGCCATCATGCTGCGAGAGGAAGCCAC 750
 T  A  L  R  D  N  R  T  T  R  R  F  Y  D  S  G  A  I  M  L  R  E  E  A  T    168

TGTCCTCACAGGGATGCTGATCGGACTCAGCGCTATCGACTTCAGCTTCTGTCTAAAGGGCGAAGTTCTGGACGG 825
 V  L  T  G  M  L  I  G  L  S  A  I  D  F  S  F  C  L  K  G  E  V  L  D  G    193

GAAGACGCCGGTGGTCATCGATTACACACCCTACCTAAAATTCACCCAAAGCTACGACTACCTGACGGATGAGGA 900
 K  T  P  V  V  I  D  Y  T  P  Y  L  K  F  T  Q  S  Y  D  Y  L  T  D  E  E    218

GGAGAGGCACAGTGCAGAGAGCAGCACCAGCGAGGACAACTCACCAGAACACCCCTACCTGCCTCTCGTCACCGA 975
 E  R  H  S  A  E  S  S  T  S  E  D  N  S  P  E  H  P  Y  L  P  L  V  T  D    243

TGAAGACAGCTGGTACAACAAGTGGCACAAGACGGAACAGAAGTTTCGCATTGTCTACGCTCAGAAGGGATACCT 1050
 E  D  S  W  Y  N  K  W  H  K  T  E  Q  K  F  R  I  V  Y  A  Q  K  G  Y  L    268

GGAGGAGCTGGTGCGTCTGCGGGAGTCGCAGCTGAAGGACCTGGAGGCGGAGAACCGGCGGCTGCAGCTGCAGCT 1125
 E  E  L  V  R  L  R  E  S  Q  L  K  D  L  E  A  E  N  R  R  L  Q  L  Q  L    293

GGAGGAGGCAGCGGCACAAAATCAGCGTGAGAAGCGGGAGCTGGAAGGAGTGATCCTGGAGCTGCAGGAGCAGCT 1200
 E  E  A  A  A  Q  N  Q  R  E  K  R  E  L  E  G  V  I  L  E  L  Q  E  Q  L    318

GACAGGTCTGATCCCCGGTGACCATGCCCCCCTGGCCCAGGGTTCCAAGGAGCTCACCACAGCCCTGGTCAACCA 1275
 T  G  L  I  P  G  D  H  A  P  L  A  Q  G  S  K  E  L  T  T  A  L  V  N  Q    343

GTGGCCCTCCCTGAGCACACTCAGTAGACCGGAGGGTGCCAGCAACTCCAAGCTATTTCGGAGACACAGCTTCAT 1350
 W  P  S  L  S  T  L  S  R  P  E  G  A  S  N  S  K  L  F  R  R  H  S  F  M    368

GAGTACGGAGCCCCTGTCTGCAGAGGCCAGCCTGAGCTCAGACTCCCAGCGCCTGGGTGAGGGCAAGAGGGACGA 1425
 S  T  E  P  L  S  A  E  A  S  L  S  S  D  S  Q  R  L  G  E  G  K  R  D  E    393

GGAACCCTGGGGCCCCATCGGGAAGGACCCCACGCCCTCCATGCTGGGCCTCTGCGGCTCCCTGGCCTCCATCCC 1500
 E  P  W  G  P  I  G  K  D  P  T  P  S  M  L  G  L  C  G  S  L  A  S  I  P    418

CAGCCTGCAAGTCCCTGGCGAGCTTCAAATCCAACGAATGCCTGGTGAGCGACAGCCCTGAGGGCAGCCCAGCAC 1575
 S  L  Q  V  P  G  E  L  Q  I  Q  R  M  P  G  E  R  Q  P                      443

TCAGCCCCAGCTGAGGAGCAGCAAGGGCAATGCCAGCCCCACCTGCCAGGGGCCATGGACAACTGCCACCTTTTG 1650

AGTCCCCCACCCCAGGCCACCCTTCAAGAACAATACCCAGCTAGCCTCAGGTCCCAGCTAGCCTCGGGTCTCTAG 1725

GGAAAGACAACCTGAGCCTCTTTCCTGCTTCAGCTTCCTGCCAAAGAGGCAGGAGCTACATGGGGAAGTGGTGGG 1800

GCCAGGAGGGACGCCCAGGTTACAGGTCGTCAGTCCCCTGGGGAAGCCACTCCATTCCGTGGTCCTGAAGGCTGC 1875

CTGGTTCCTTCTGTTCATCTTCCACACTTGCCTCAGAAGCAGGTGGTCCAGCCCTGGCATTCCTGCTGCCCTGCC 1950

TCTGGTCTAACCCTGTGTACCCTCTGAAGTCACCCTTCCTCGGTACCTATGTGGGGAGAGATTAGGCA AATAAAA 2025

ACCAGAGGACTGAAAAAAAAAAAAAAAAAAA                                              2100
```

Figure 3.12 A schematic representation of how clones OL0755-A, OL0755-B and OL0755 are related at the nucleotide level, possibly by alternative splicing of a common gene. The figures are drawn to scale with respect to clone sizes and blocks of sequence indicated by solid black represent sequence which is unique and specific to each clone. Hence clone OL0755-A has 3 unique segments of sequence, including one segment of about 600 bp in size, and clone OL0755-B has one unique segment of sequence. Blocks of sequence with the same shade of grey represent identical sequence common to the clones. The thick black lines under clones OL0755-A and OL0755-B indicate the locations of clone OL0755 sequence. The locations of the start and stop codons giving rise to the encoded proteins are also indicated for clones OL0755-A and OL0755-B. Hence the proteins encoded by clones OL0755-A and OL0755-B have identical N-termini but different C-termini.

Clone OL0755-A



Clone OL0755-B



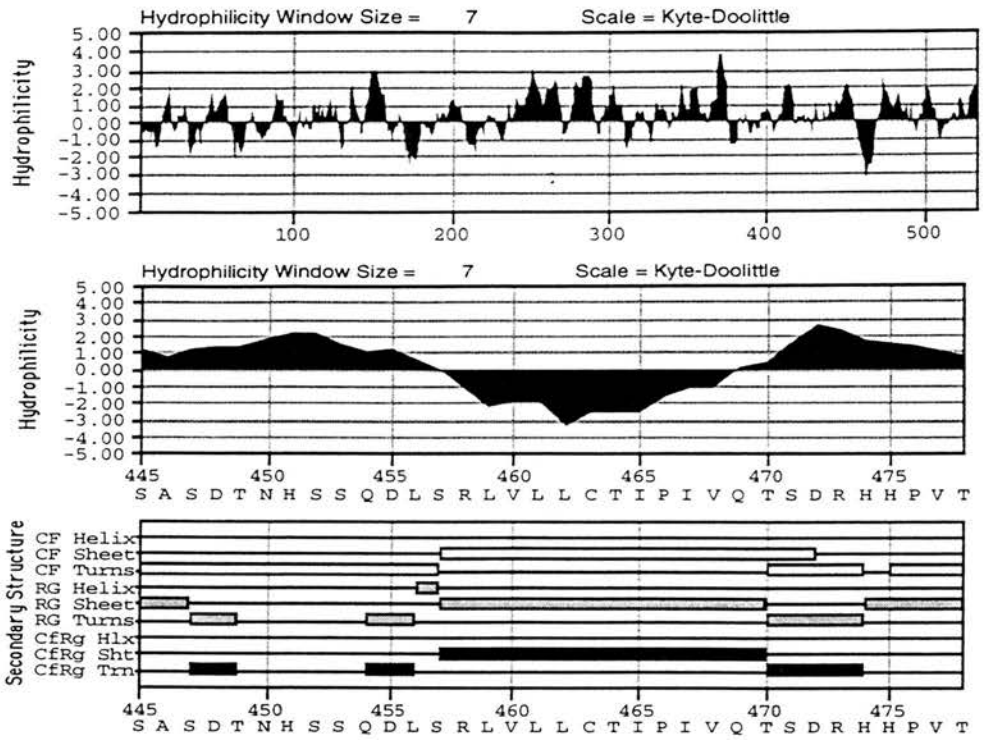
Clone OL0755

Figure 3.13 Hydrophilicity plots for the proteins encoded by clones OL0755-A and OL0755-B.

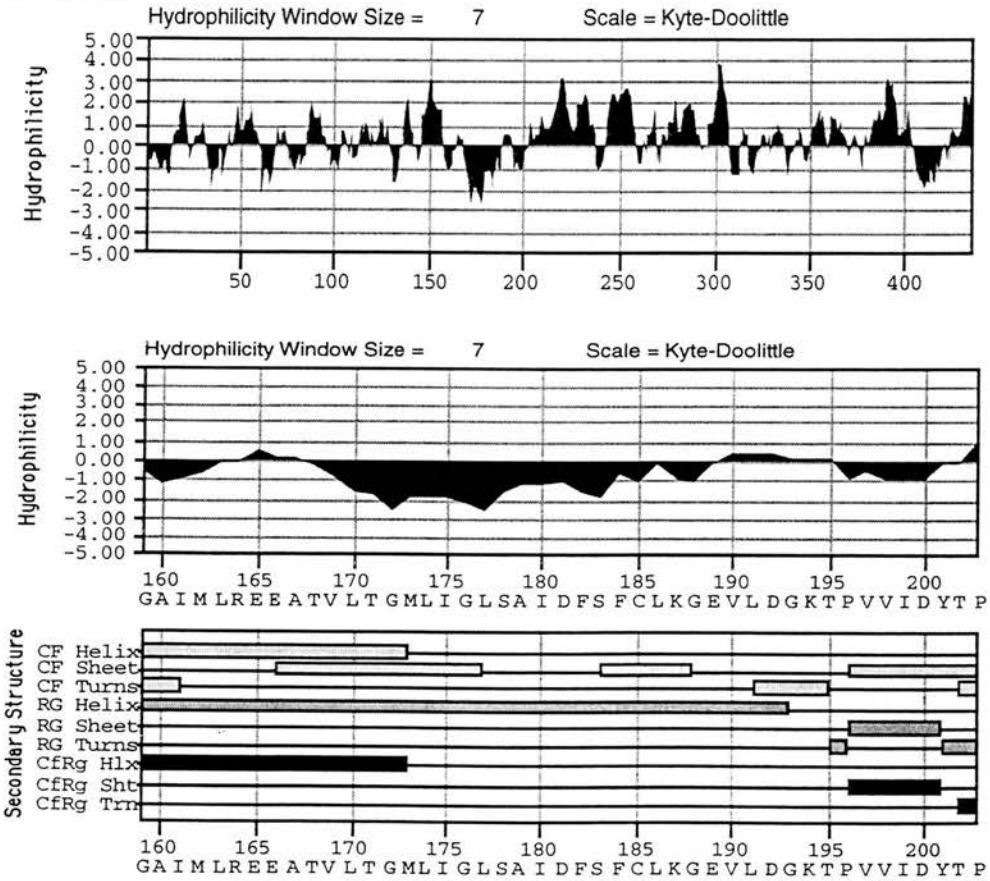
Clone OL0755-A is mostly hydrophilic in nature except for the 13 amino acid stretch from Ser-457 to Gln-469 which also has predicted beta-sheet secondary structure and may therefore be a potential transmembrane domain.

Clone OL0755-B is also mostly hydrophilic except for the region from Thr-168 to Glu-188 which has possible alpha helix structure and so may also be a potential transmembrane domain.

Clone OL0755-A



Clone OL0755-B



identified at amino acids 199 and 231. The glycosylation sites present in both clones, however, are apparently not used (see 3.4) supporting the view that these clones do not encode integral membrane proteins.

The only significant similarity of these clones to any other sequence in the Genbank or EMBL databases is a 95.6% identity of 201 nucleotides (bp 1956-2157 in clone OL0755-A, which is also present in clone OL0755-B) with a R. norvegicus partial brain mRNA named clone sap37f. This clone was isolated from an expression cDNA library with antisera raised against a synaptic protein preparation, the postsynaptic density fraction (Langnaese et al., 1996).

Conclusions and mini discussion

During hybridisation screening of the library, PCR amplification was a useful way to confirm that clones were present which were related to clone OL0755. Amplification with the primers specific to clone OL0755, 11F and 7R, was a reliable reaction. PCR amplifications to estimate the amount of additional 5′ sequence present in clones related to OL0755 was not as reliable probably because conditions were not optimal for many of the template clones.

Two related clones, OL0755-A and OL0755-B, were isolated from this library. Clone OL0755-A was identified by digoxigenin labelled clone OL0755 and clone OL0755-B by ^{32}P radiolabelled probe. Digoxigenin labelling was initially used as an alternative to radioactivity and therefore as way to avoid the additional hazards associated. However it does appear to be less sensitive since from six plates in a secondary hybridisation screen only eight positive plaques were identified with a digoxigenin labelled probe compared to 17 positive plaques from only four plates when a radiolabelled probe was used. Also, when comparing plates 2 and 3 which were analysed by both methods, three positive plaques were identified on plate 2 and one positive on plate 3 by the digoxigenin labelled probe as opposed to six and three positive plaques identified with radioactively labelled probe.

The sizes of clones OL0755-A and OL0755-B compare well with the two mRNAs identified on the Northern blot and sequence analysis shows their 3′ ends to be complete with polyadenylation signal and poly A tail. Therefore it appears that both clones are full length cDNAs. Sequence analysis predicts that clone OL0755-A encodes a protein of about 60 kD and clone OL0755-B one of about 50 kD. Both proteins are mostly hydrophilic but do contain potential membrane spanning domains, although since the glycosylation sites present in the proteins are apparently not used (see 3.4) it seems unlikely that these proteins are integral membrane proteins. A small, 201 bp fragment present in both clones is almost identical to a

region of a clone, sap37f, isolated from an expression cDNA library with antisera raised against a synaptic protein preparation, the postsynaptic density fraction (Langnaese et al., 1996). It is possible therefore that the proteins encoded by OL0755-A and OL0755-B could be associated with synaptic elements of the CNS.

Sequence analysis shows that clones OL0755-A and OL0755-B are closely related and are possibly the products of alternative splicing of a primary transcript encoded by a common gene. However, the relationship of these two clones with clone OL0755 is less straightforward. It is possible that clone OL0755 represents the partial sequence of a third related clone. If this were the case, however, explaining the relationship of all three clones by alternative splicing mechanisms would be difficult. It is perhaps more probable that clone OL0755 is a truncated form of clone OL0755-B resulting from an error of the reverse transcription of polyA RNA during the construction of the original cDNA library (see chapter 4 for a more detailed discussion).

3.4 Expression patterns of clones OL0755-A and OL0755-B

3.4.1 Northern blot analysis with a clone OL0755-A specific probe

Clone OL0755-A has a 624 bp fragment of sequence (nucleotides 1286-1910) which is not present in clone OL0755-B and therefore is ideal for use as a probe specific for clone OL0755-A. PCR amplification using primers 16F (5'GATCATGAGGACATC3') and 21R (5'AGAGGGAAGCAAGTCTCAGC3') was used to isolate a 534 bp fragment from this region (see Figure 3.10). Figure 3.14 shows a 1% agarose gel with the 534 bp amplified product. The negative control which had no template DNA is clear giving confidence that the DNA of interest was amplified and not an artefact. Ligation of the PCR product into the TA vector was followed by sequencing of the insert with the T7 and M13F primers to confirm that the sequence was correct. This isolated fragment specific to clone OL0755-A will be called clone OL0755-A(S).

A Northern blot experiment was done using clones OL0755 and OL0755-A(S) to probe 15 day old rat brain total RNA (15 μg). Plasmid DNA for clones OL0755 and OL0755-A(S) were radioactively labelled with [α-^{32}P] dCTP by random priming and hybridised with the filters at 65^{o}C. Autoradiography of the filters (Figure 3.15) showed that clone OL0755 hybridised with at least two mRNAs as shown before but clone OL0755-A(S) hybridised specifically to the 3 kb mRNA which provides good evidence that clone OL0755-A correlates to this mRNA.

3.4.2 Developmental Northern blot

A developmental Northern blot was done using clone OL0755 to probe equal amounts of total RNA from rat brains of different ages to give an indication of the levels of expression during postnatal development of the central nervous system. Total RNA was isolated from whole brains of rats aged 1, 3, 5, 7, 9, 11, 15, 21days and adult (16 weeks old) and an equal amount (10 μg) of each was run on a 0.8% formaldehyde agarose gel and transferred onto a nylon membrane. Figure 3.16 shows the autoradiograph of the filter with equal loadings of these different aged rat brain RNAs after being hybridised with clone OL0755. The 2 mRNAs of about 2 kb and 3 kb are clearly identified at every age examined. Expression of the smaller mRNA increases over time during development until postnatal day 21. The larger 3 kb mRNA is not as abundant as the smaller one but increases in amount with developmental age until P21, although not as dramatically. There may be a third mRNA band present at 4 kb although it is difficult to discern it from non-specific signal associated with the large ribosomal subunit.

Figure 3.14 PCR amplification of clone OL0755-A(S), the 534 bp fragment specific to clone OL0755-A. Primers 16F and 21R were used to amplify up this region of the clone. The negative control with no added template DNA is clear.

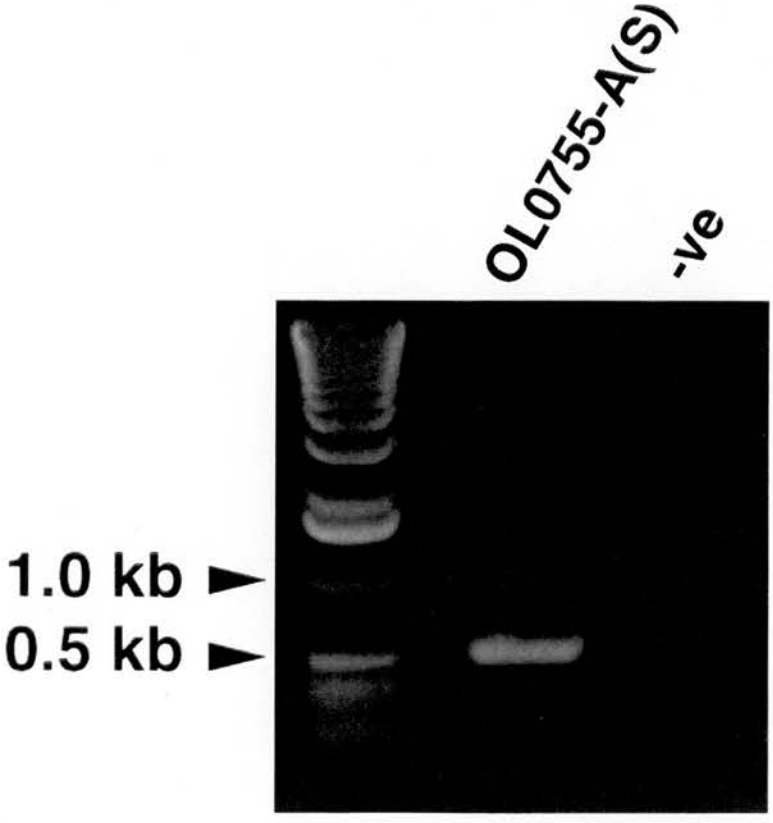

OL0755-A(S)
-ve
1.0 kb
0.5 kb

Figure 3.15 Northern blot of 15 day-old rat brain total RNA probed with clones OL0755 and OL0755-A(S). The autoradiograph clearly shows that probe OL0755-A(S) is specifically hybridising with the mRNA of about 3 kb recognised by clone OL0755. Hence this band correlates well with clone OL0755-A (2.7 kb).

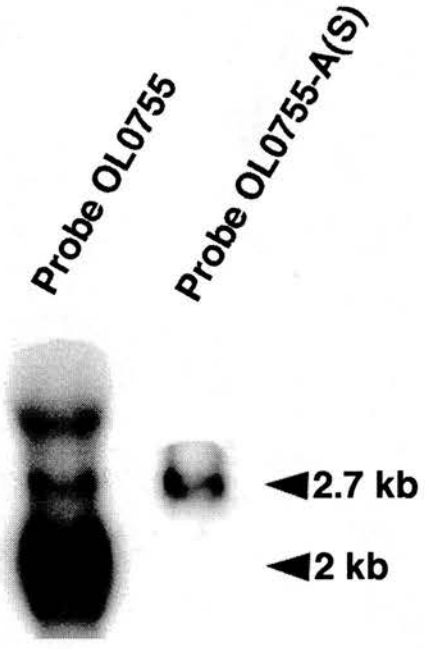
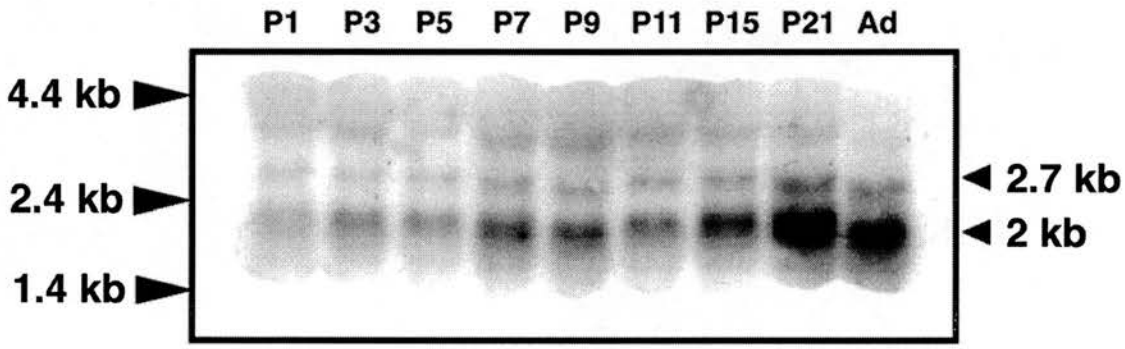

Probe OL0755
Probe OL0755-A(S)
2.7 kb
2 kb

Figure 3.16 A developmental Northern blot to investigate expression levels of clone OL0755 related mRNAs during development. Total RNA (10 μg) from rat brains of different ages were run out on a 0.8% formaldehyde agarose gel, transferred onto Hybond-N nylon membrane and hybridised with radiolabelled probe OL0755. The resultant autoradiograph clearly shows an increase in expression of both the 2.7 kb and 2 kb mRNAs, correlating with clones OL0755-A and OL0755-B respectively, during development until postnatal day 21 (P21).


P1
P3
P5
P7
P9
P11
P15
P21
Ad
4.4 kb
2.4 kb
1.4 kb
2.7 kb
2 kb

3.4.3 Production of a polyclonal antibody against an N-terminus peptide

Clones OL0755-A and B have common N-terminus amino acid sequence. A polyclonal antibody was raised against a 23 amino acid peptide at the extreme N-terminus. The peptide sequence was EASFVQTTMALGLPSKKASSRNV. A reactive antibody was produced which recognises a single protein with a similar size to actin (the control reaction), as seen in Figure 3.17. Actin is approximately 43 kD in size and therefore it is possible that the protein being identified by the anti-peptide antibody is that encoded by clone OL0755-B with a predicted size of about 50 kD. The full bleed sample clearly recognises the same proteins as the test bleed and there is little evidence of background signal although the titre of the antibody does appear to have decreased slightly. There is a very faint band above the stronger one which may correspond to the 60 kD (approximately) protein encoded by clone OL0755-A. The abundance of this clone is less than for clone OL0755-B as shown by the developmental Northern blot so it seems no surprise that the encoded protein may also be less abundant than that encoded by clone OL0755-B. This antibody will be called Ab755 since it was raised against a peptide common to both clones OL0755-A and B just as clone OL0755, when used as a probe, recognises both.

3.4.4 Developmental Western blot with Ab755

Western blotting of brain homogenates from rats of different ages with Ab755 was used to investigate expression of the proteins encoded by clones OL0755-A and B during development. Equal amounts of protein (100 μg) for each brain homogenate sample were loaded onto a 10% SDS PAGE gel which separated the proteins according to size. The proteins were then transferred onto a nitrocellulose membrane which was incubated with Ab755 at a dilution of 1:500. Figure 3.18 shows the developmental Western blot. Expression of the small 50 kD protein is increasing significantly with developmental age until postnatal day 21. This pattern compares well with the developmental expression of the mRNA encoding clone OL0755-B (see the developmental Northern blot). The larger 60 kD protein is much less abundant and expression appears to decrease slightly with age unlike expression of the mRNA which increases slightly.

3.4.5 ISH studies on rat brain sagittal sections

Attempts were made to use Ab755 in immunofluoresence studies on rat brain sagittal sections to investigate expression patterns of the proteins in vivo. However these proved to be unsuccessful. This may possibly be due to the native proteins having a conformation which does not allow access by the antibody to the correct

Figure 3.17 Test Western blots with Ab755 against rat brain homogenate. Strips of a filter on which the proteins of rat brain homogenate were separated were incubated with Ab755 at two dilutions (1:500 and 1:1000). Both the antisera from the test bleed and the full bleed are clearly reacting with a protein a little larger than actin (43 kD) which may correlate with the 50 kD protein encoded by clone OL0755-B. There is also a weaker reaction with a larger protein which may correlate with the 60 kD protein encoded by clone OL0755-A. Unfortunately the titre of Ab755 has decreased from the test bleed to the full bleed.

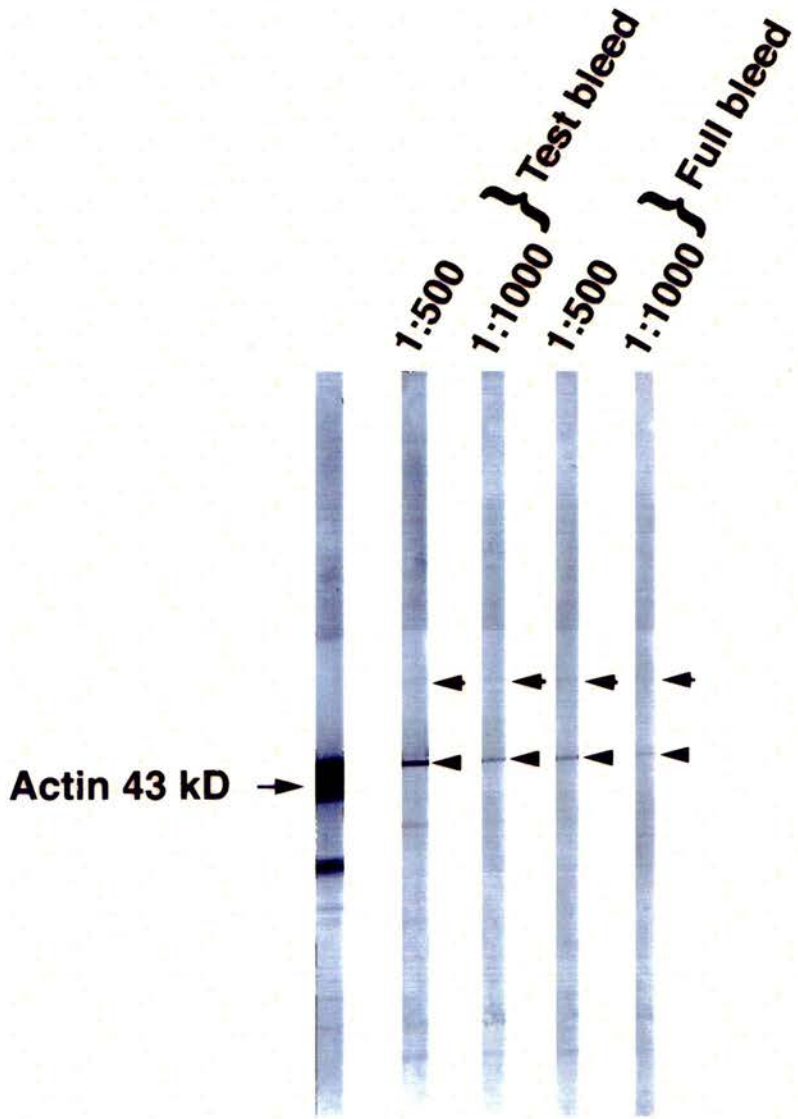
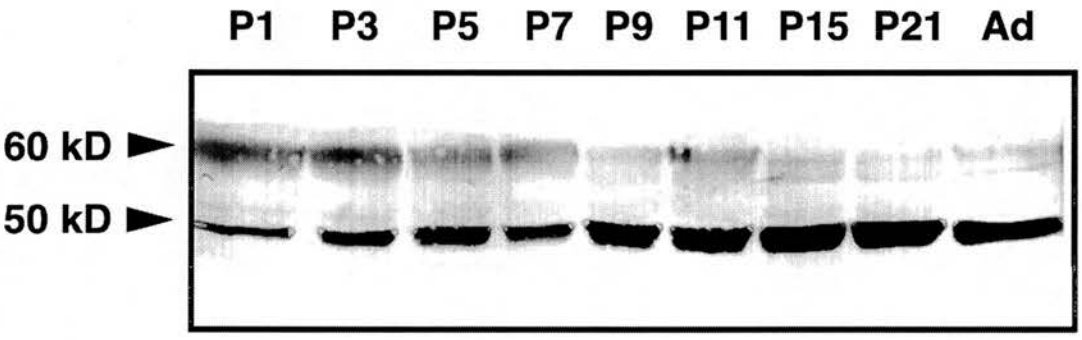

1:500
1:1000
Test bleed
1:500
1:1000
Full bleed
Actin 43 kD

Figure 3.18 Developmental Western blot to investigate expression of the proteins encoded by clones OL0755-A and OL0755-B during development. Protein (100 μg) from brain homogenates of different aged rats were run on a 10% SDS PAGE gel, transferred onto a nitrocellulose membrane and incubated with Ab755 diluted 1:500. Expression of the 50 kD protein encoded by clone OL0755-B is increasing with age until postnatal day 21 (P21). Expression of the 60 kD protein encoded by clone OL0755-A decreases during development.


P1
P3
P5
P7
P9
P11
P15
P21
Ad
60 kD
50 kD

epitope. Therefore further attempts were made following treatment of sections with SDS according to the method of Miner et al., 1997, in the hope that these denaturing conditions might expose the epitope on the proteins which reacts with Ab755. Again, however, this approach proved unsuccessful. Therefore ISH studies were performed as an alternative in order to investigate expression patterns in the rat brain of the mRNAs encoding the proteins.

ISH employed clone OL0755 as a riboprobe since its sequence is common to both clones OL0755-A and OL0755-B and 21 day-old rat sagittal brain sections were used since this age corresponds to the maximum expression of both the clones as deduced from the developmental Northern blot. Immunofluoresence with antibodies against GFAP to identify astrocytes and against MBP to identify oligodendrocytes was also performed on the same sections in an attempt to investigate whether clone OL0755 was expressed by a specific cell type in vivo and if this expression correlated with expression in cultured oligodendrocytes and astrocytes. Unfortunately morphological preservation of these cell types was not good.

Figure 3.19 shows the results of ISH of clone OL0755 on 21 day-old rat brain sagittal sections. It is highly expressed in cells of the granule cell layer in the cerebellum. At higher magnification expression is seen in the cerebellar nuclei and possibly also in some cells of the molecular layer. There is strong expression in the hippocampal CA1, CA2 and CA3 regions as well as in the dentate gyrus, and throughout the fore-brain there is also expression. The labelling in the granule cells of the cerebellum, the cerebellar nuclei and the hippocampus strongly indicate expression of clone OL0755 by neurons, however MBP and GFAP antibody staining in the fore-brain region seems to correlate in some cells with ISH labelling by clone OL0755 (as shown more clearly in Figure 3.20) and therefore suggests expression by glial cells as does the possible labelling of some cells in the molecular layer of the cerebellum.

ISH was also done on 21 day-old rat brain sagittal sections with clone OL0755-A(S) as the riboprobe to see if there is a different pattern of expression for clone OL0755-A alone. Figure 3.21 shows the results. Clone OL0755-A apparently has a similar pattern of expression to clone OL0755, although perhaps not as strong. It strongly labels cells of the granule cell layer in the cerebellum and the hippocampus and dentate gyrus but does not seem to be as abundantly expressed in fore-brain as clone OL0755.

Figure 3.19 ISH of 21 day-old rat brain sagittal sections with clone OL0755. Panel A shows the complete sagittal brain section indicating ISH labelling in the cerebellum (CB), hippocampus (HP) and fore-brain (FB). Panels B, C and D show ISH labelling in the granule cell layer (GL) of the cerebellum. Panel C also shows labelling of the cerebellar nuclei (CN). Panel E shows labelling in the hippocampus. The regions CA1, CA2 and CA3 are indicated, as is the dentate gyrus (DG). Panel F shows ISH labelling in the granule cell layer of the cerebellum by epifluorescence. Labelling of some cells in the molecular layer (ML) is indicated by the arrow. MT=myelinated tract.

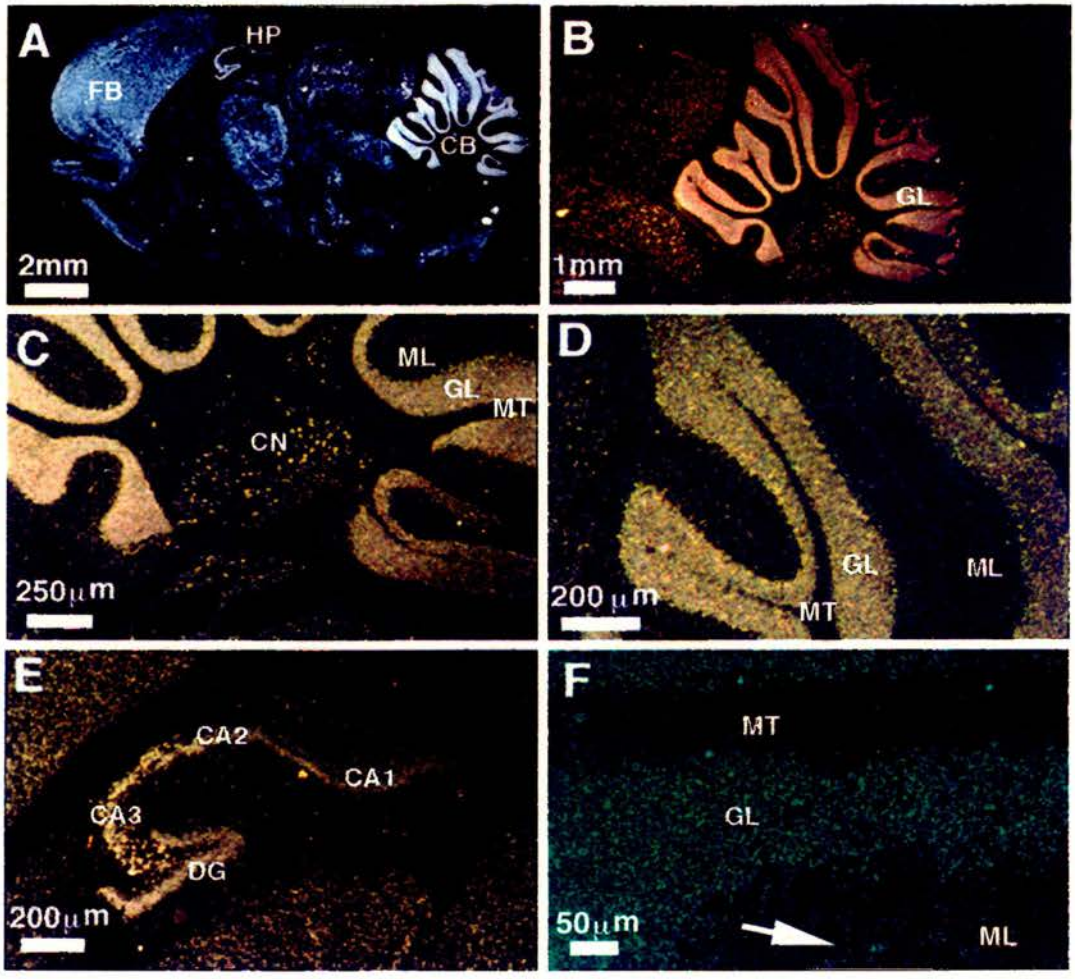

A
HP
FB
CB
2mm
B
GL
1mm
C
ML
GL
MT
CN
250μm
D
GL
ML
200μm
MT
E
CA2
CA1
CA3
DG
200μm
F
MT
GL
50μm
ML

Figure 3.20 21 day-old rat brain sagittal section immunologically labelled with antibodies against GFAP and MBP and ISH with clone OL0755. Panel A shows immunocytochemical labelling of astrocytes in the forebrain with the anti-GFAP monoclonal antibody GA5. There is a correlation of some cells with ISH labelling by clone OL0755, panel B, indicated by an asterisk. ISH labelling of other cells shown in panel B correlate with oligodendrocytes labelled by an anti-MBP antibody shown in panel C, an example of which is indicated by an arrow.

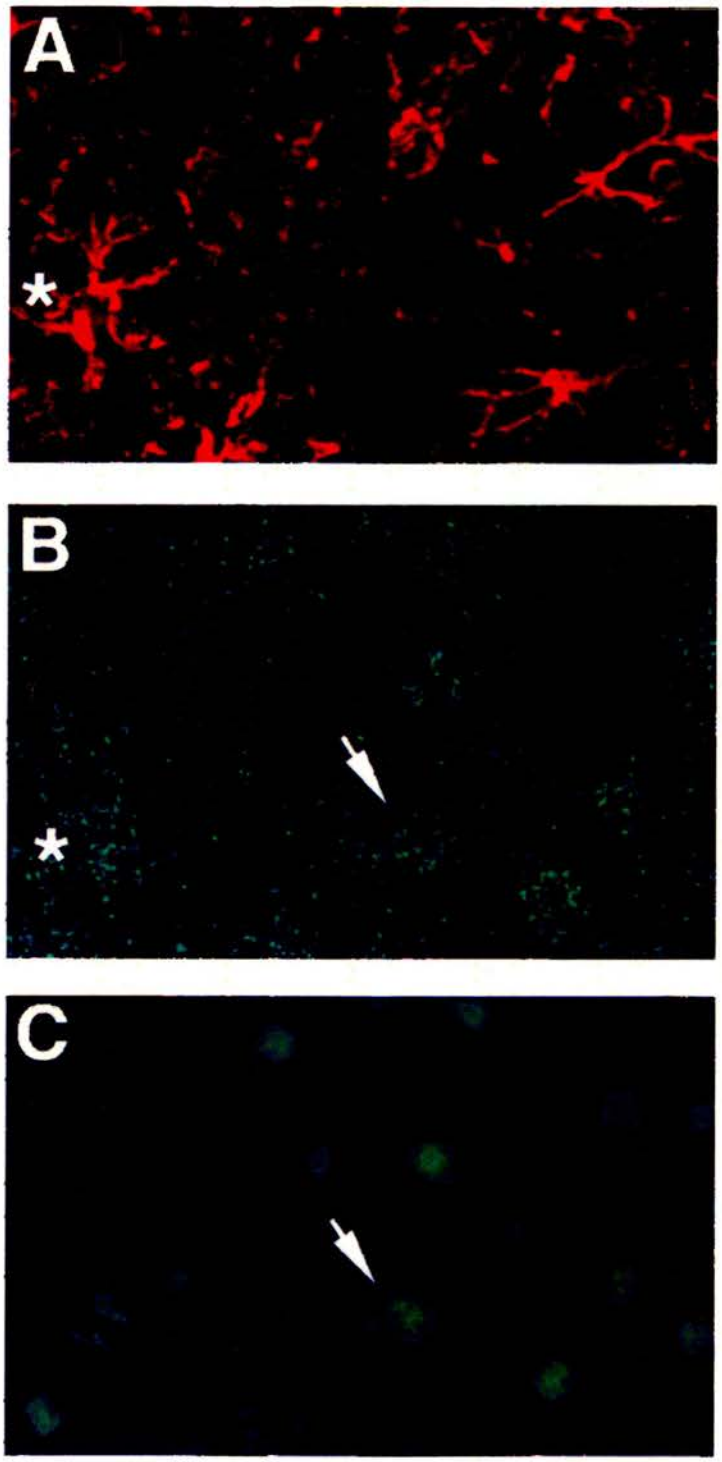
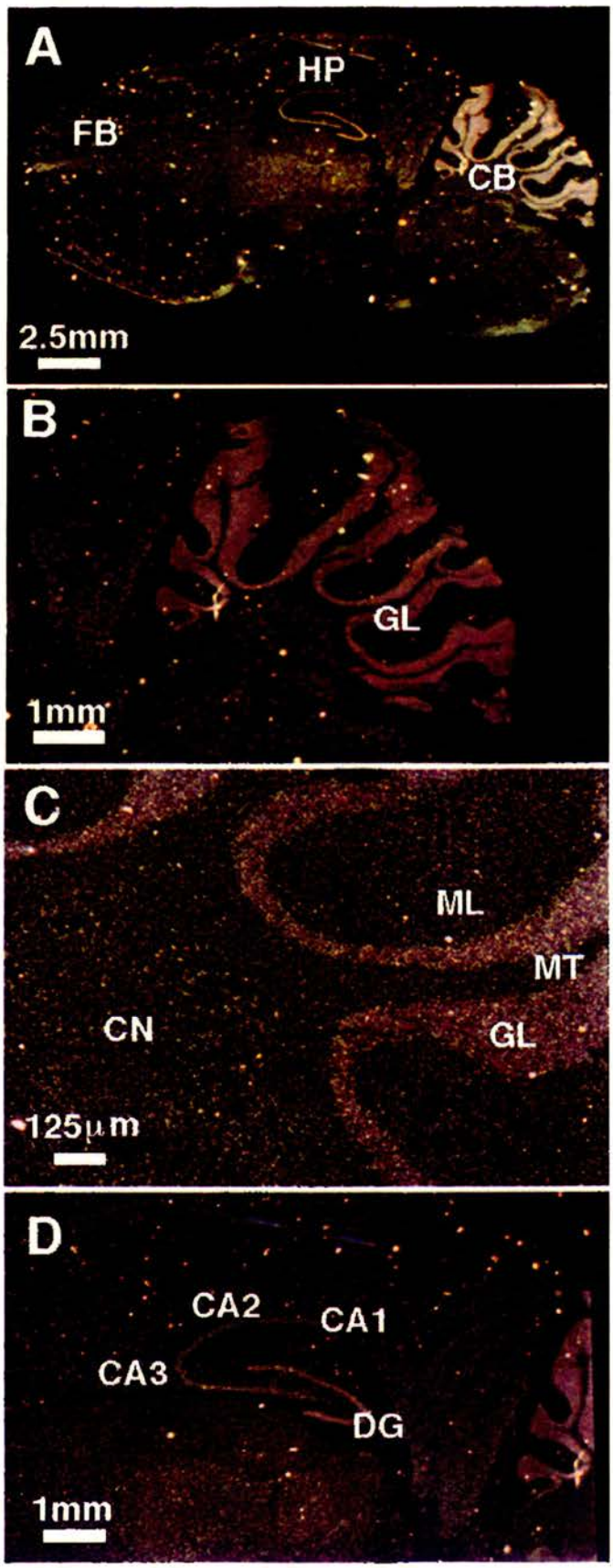

A
*
B
*
C

Figure 3.21 ISH of 21 day-old rat brain sagittal sections with clone OL0755-A(S). Panel A shows the complete sagittal brain section indicating ISH labelling in the cerebellum (CB) and in the hippocampus (HP). Panels B and C both show granule cell layer (GL) labelling in the cerebellum. There also appears to be weak staining in the cerebellar nuclei (CN) in panel C. Panel D shows labelling of the hippocampal CA1, CA2 and CA3 regions, as well as the dentate gyrus (DG). FB=fore-brain, ML=molecular layer, MT=myelinated tract.


A
HP
FB
CB
2.5mm
B
GL
1mm
C
ML
MT
CN
GL
125μm
D
CA2
CA1
CA3
DG
1mm

3.4.6 ISH studies on rat optic nerve sections

As previously described, the optic nerve is the simplest part of the CNS and does not contain any neuronal cell bodies. It was therefore decided to do an ISH study with clones OL0755 and OL0755-A(S) on 21day rat optic nerve transverse and longitudinal sections in the hope of confirming expression by glial cells. Figure 3.22 shows the results. There is clear labelling of the optic nerve by clones OL0755 and OL0755-A(S) antisense riboprobes as compared with the sense controls. Therefore it can be deduced that the mRNAs for these clones are expressed by glial cells .

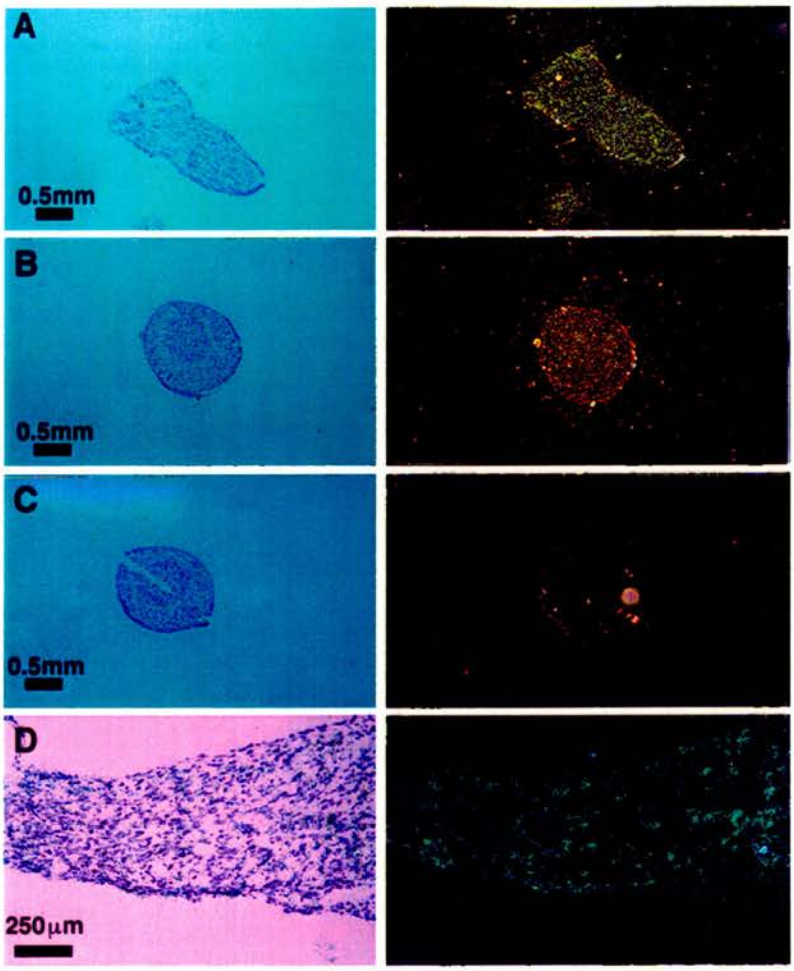
3.4.7 Investigations to deduce whether the proteins encoded by clones OL0755-A and B are phosphorylated and/or glycosylated

Figure 3.23 shows samples of brain homogenate from 5 day and 21 day-old rats which had been treated with and without dephosphatase enzyme and then run on a 10% SDS PAGE gel, transferred onto a nitrocellulose membrane and incubated with Ab755. A shift in the mobility of the proteins identified by Ab755 compared with untreated proteins would be indicative that they had been dephosphorylated by the enzyme and that in vivo they are phosphorylated. However it appears that treatment of these proteins with the dephosphatase enzyme has not caused them to run differently on the gel by comparison with the untreated proteins. This lack of mobility shift does not however prove that the proteins are not phosphorylated. It is only possible to state that a shift in mobility confirms phosphorylation of the native proteins but no shift in mobility does not exclude this possibility.

The same 5 day and 21 day-old rat brain homogenates were also treated with the de-glycosylation enzyme N-Glycosidase F. Glycosylation of a protein is indicative of localisation to the plasma membrane of a cell. The deglycosylated form of a normally glycosylated protein has an increased mobility on an SDS PAGE gel compared with untreated protein as can be seen in Figure 3.24 with MAG. Brain homogenate which had been treated with and without N-Glycosidase F was incubated with an antibody against MAG (diluted at 1:2000). This acted as a control for the deglycosylation reaction and it can clearly be seen that there is an increase in the mobility of MAG on the gel following deglycosylation. The diffuse band of the native glycosylated protein changes to a sharper band following deglycosylation. However, it appears that the proteins recognised by Ab755 are not glycosylated in their native form since there is clearly no shift in mobility for either protein on the gel.

Figure 3.22 ISH of 21 day-old rat optic nerve sections with clones OL0755 and OL0755-A(S). Bright field images are shown on the left and dark field images on the right of each pair. Panels A show longitudinal sections and panels B transverse sections of the nerve labelled with antisense riboprobes for clone OL0755-A(S) and OL0755 as indicated. Panels C show transverse sections hybridised with sense control riboprobes for each clone. Clone OL0755-A(S) and OL0755 both clearly label the optic nerve by comparison to the sense controls, although labelling by clone OL0755 is slightly stronger than by clone OL0755-A(S). Panel D shows labelling of a transverse section of nerve by clone OL0755, viewed by epifluorescence. Clusterings of black silver grains are also apparent in the bright field image.

Clone OL0755



Clone OL0755-A(S)

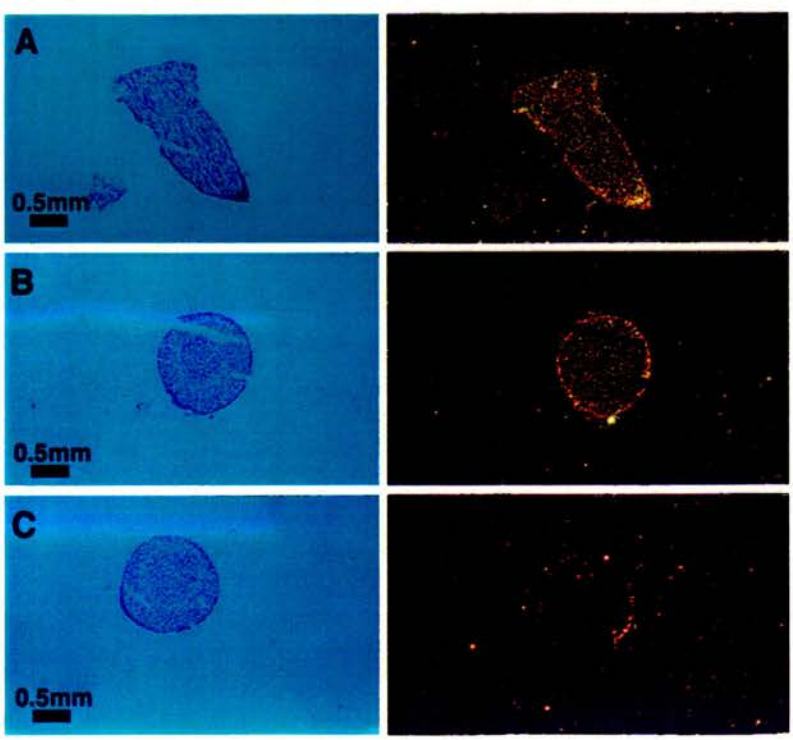


Figure 3.23 Neither protein encoded by clones OL0755-A (60 kD) or OL0755-B (50 kD) change their electrophoretic mobility following dephosphorylation. Brain homogenates from 5 day and 21 day rat brains were incubated with (+) or without (-) λphosphatase and then run on a 10% SDS PAGE gel before transfer to a nitrocellulose membrane and incubation with Ab755 diluted 1:500. A shift in mobility would confirm phosphorylation of the native proteins but an absence of a shift does not exclude the possibility.

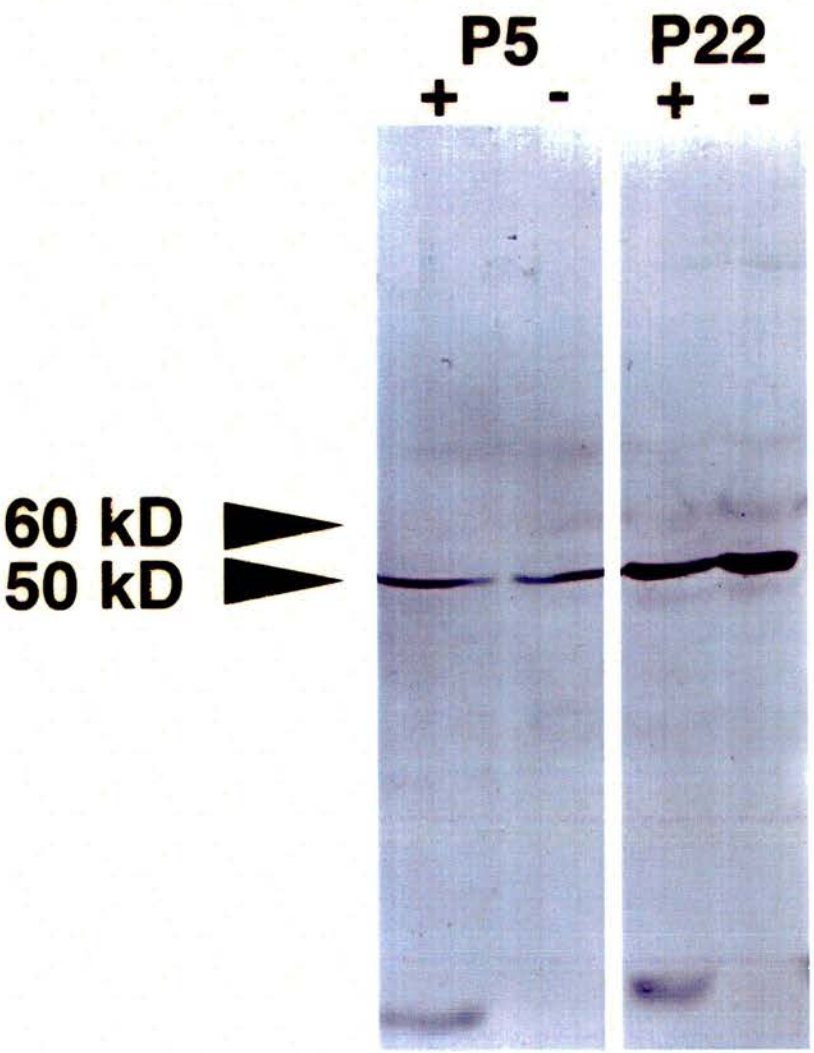
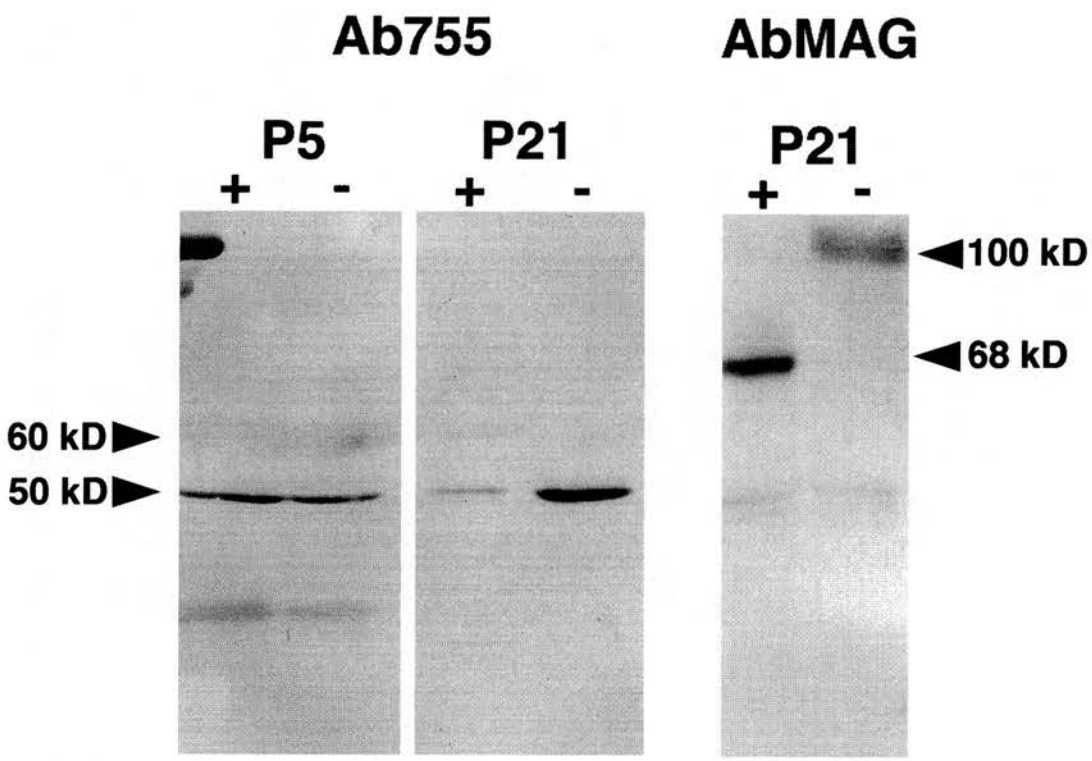

P5
P22
+
-
+
-
60 kD
50 kD

Figure 3.24 Neither protein encoded by clones OL0755-A (60 kD) or OL0755-B (50 kD) change their electrophoretic mobility following deglycosylation. Brain homogenates from 5 day and 21 day rat brains were incubated with (+) or without (-) N-glycosidase F (as described in Materials and Methods) and then run on a 10% SDS PAGE gel before transfer to a nitrocellulose membrane and incubation with Ab755 diluted 1:500. An increase in mobility would confirm the presence of N-linked oligosaccharide chains in the native proteins. This can be seen with MAG where cleavage of the N-linked oligosaccharides has resulted in a large increase in mobility.


Ab755
AbMAG
P5
P21
P21
+
-
+
-
+
-
60 kD
50 kD
100 kD
68 kD

Conclusions

The Northern blot with a probe specific to clone OL0755-A demonstrates that this clone directly correlates with the mRNA of about 3 kb identified by clone OL0755. It was not possible to repeat this experiment with a probe specific to clone OL0755-B since it only contains a small 50 bp segment which is not also present in clone OL0755-A.

The anti-peptide antibody generated against the N-terminus, common to both encoded proteins, reacted with two proteins a little larger than actin (43 kD). Unfortunately the titre of the full bleed was a little less than in the test bleed. In a developmental Western blot the two proteins which reacted with Ab755 were of a size which correlated well with that predicted from sequence analysis of clones OL0755-A and OL0755-B. This gave support to the notion that clones OL0755-A and OL0755-B were full length cDNAs.

Both the mRNAs and encoded proteins are developmentally regulated with peak expression at P21. The smaller clone and associated protein is consistently less abundant than the larger throughout development.

It was not possible to investigate protein expression patterns but mRNA expression in P21 rat brain is predominantly neuronal with some expression in glial cells which was confirmed by ISH studies on optic nerve. Neither protein appears to be phosphorylated or glycosylated so it is difficult to assign a particular role for these proteins although their developmental profile and specificity to the CNS suggest a potential role in nervous system development.

CHAPTER 4. DISCUSSION

4.1 Was subtractive hybridisation a successful approach to identifying novel components involved in the development of the nervous system?

Subtractive hybridisation is a powerful technique for identifying genes which demonstrate cell type or developmental stage specific expression (Watson and Marguiles, 1993). A glial progenitor cDNA library was used to remove common sequences from a differentiated glial library. The remaining sequences were subcloned to prepare the subtracted cDNA library comprising clones which are specific to differentiated glia. The complexity of this library was approximately 300,000. Many of the known sequences identified included the well characterised myelin proteins such as MBP and PLP (see Table 3.1) These proteins are expressed by differentiated glia and therefore indicate that the subtraction process has been successful in screening out sequences also expressed by progenitor cells. Of the 1003 clones analysed, 86 proved to be novel when their partial sequences were compared with the EMBL and Genbank databases. This is 8.6% of the clones analysed. Five clones, 0.5% of the total, were novel and specific to the nervous system. A particularly important feature of subtractive hybridisation is that it greatly enhances the probability of isolating sequences encoding rare mRNAs which are the ones most likely to be cell specific. It has been suggested that this technique is able to detect transcripts with an abundance of less than 0.001% in an mRNA population in vivo (Ausubel et al. 1992). Therefore the five brain specific clones isolated, with an abundance of 0.5% of the total number of clones analysed, are likely to be rare mRNAs present in differentiated glia which have been enriched for during the subtraction process and indicate that the subtractive hybridisation approach was a successful means of identifying novel genes possibly involved in nervous system development. They also indicate that the differentiated glial subtractive library is a rich resource of additional novel nervous system clones for future investigations.

4.2 Deductions about the five novel clones from Northern blots and ISH studies on cultured glial cells.

When the expression patterns of the five novel cDNA clones were examined by Northern blotting they all identified mRNAs which were large in size, except clone OL0755 which was also the only clone to identify more than one mRNA. Two clones were expressed in both the PNS and CNS indicating expression by a cell type(s) common to both such as neurons or myelinating glia. Clone OL0816 was only expressed in brain. It was not detected in either optic nerve or sciatic nerve, so this indicates possible expression by a subset of neurons present only in brain tissue. Clones OL0755 and OL0848 both showed a pattern of expression specific to the

CNS. Signal detected in optic nerve indicates expression by glial cells i.e. oligodendrocytes and/or astrocytes. Since it is generally accepted that neuronal axons do not contain mRNAs and there are no neuronal cell bodies in optic nerve, signal in optic nerve on the Northern blot cannot arise from expression by neurons. Northern blot results therefore indicate that clones OL0755 and OL0848 are expressed by neurons and/or glial cells.

Clone OL0003 appears to demonstrate an upregulation in expression from P1 to P10. In the rat CNS P1 corresponds to a time just before oligodendrocytes first appear and begin to myelinate nerve axons. This time would also correspond to the switching on of genes required for the process of myelination. By P10 myelination is well underway and there is generally high expression of proteins involved such as MBP and PLP etc. There is considerably more than residual expression of clone OL0003 at P1 so it appears unlikely that this clone is expressed exclusively by differentiated glia, unlike MBP. The expression pattern for MBP (not shown) demonstrates no signal at all at P1 and intense signal at P10 corresponding to the change from undifferentiated progenitor cells present at around the time of birth and which do not express MBP, to differentiated oligodendrocytes expressing MBP as they synthesise myelin.

Clone OL0003 was subsequently proved to encode the 3′-untranslated region of neurofascin by M. Collinson. This protein is a member of a subgroup of the Ig superfamily called the L1CAMS and has been implicated in neurite outgrowth, fasciculation and inter-neuronal adhesion (Grumet et al., 1991; Rathjen and Schachner, 1984; Volkmer et al., 1992). It is widely recognised as a neuronal cell adhesion molecule (CAM) but recent studies have also shown it to be expressed by oligodendrocytes (Moscoso and Sanes, 1995) as did the ISH studies carried out on cultured glial cells in this study. Neurofascin is developmentally regulated in oligodendrocytes with mRNA first detectable when the gene for PLP becomes transcriptionally active. Expression declines when ensheathment of the target axon begins, therefore neurofascin appears not to be involved in compaction or stabilisation of the myelin sheath (Collinson et al, 1997). Although expression declines in oligodendrocytes it continues to be expressed by neurons into adulthood. Neurofascin has been implicated as a candidate cell surface molecule mediating and signalling axon-glial interaction during the first stages of myelination (Collinson et al., 1997). Therefore the significant signal at P1 on the Northern blot probed with clone OL0003 is most likely due to expression of neurofascin by neurons before myelination begins. By P10 the signal is due to both neurons and oligodendrocytes which are actively interacting during the first stages of myelination. It could be

noted that the subtraction process should have removed all sequences expressed by progenitor cells and therefore no clones with expression at P1 should have been identified. However, perhaps the low abundance of these clones in the cDNA libraries could explain how they managed to avoid the subtraction process. Possibly if the subtraction had been performed more than twice, such clones would have been removed.

When expression of the five clones was analysed more closely by ISH experiments on cultured glial cells, all five clones appeared to be expressed by cells of the oligodendrocyte lineage which were labelled immunocytochemically by the monoclonal antibody 04. Clone OL0755 showed expression by both oligodendrocytes and astrocytes. This result agrees with the Northern blotting analysis in which a signal was evident in optic nerve indicating expression by glial cells. Interestingly, clone OL0816 is the only other clone to show expression by both glial cell types, although weakly. However no signal was detected in optic nerve on the Northern blot. This may reflect a difference in sensitivity between the two techniques. Alternatively the results from ISH experiments on cultured cells may be less representative of what actually occurs *in vivo*. It is possible that cells in culture are induced to express some proteins artificially. An example is MAP5, also known as MAP1B (Vouyiouklis and Brophy, 1993), which has been shown to be expressed in cultured oligodendrocytes but *in vivo* is expressed primarily in neurons (Fischer et al., 1990; Schoenfeld et al., 1989). Hence it is important to employ caution when interpreting results obtained from an *in vitro* environment and extrapolating them to an *in vivo* situation.

4.3 The relationship of clones OL0755-A and OL0755-B to OL0755.

Clone OL0755 was chosen for further investigation. Since this cDNA clone contained an insert of about 1 kb in size and yet identified mRNAs on Northern blots of around 2 and 3 kb, it could not possibly be full length. It was therefore used to screen a cDNA library to obtain related clones which could be full length. The library screened was oligo dT primed since from the partial sequence it was known that the 3′ end of clone OL0755 was complete and included a polyadenylation signal and a polyA tail. The library was also directionally cloned in the NotI to SalI direction to ensure that any related sequences identified by clone OL0755 could be correctly oriented. The cDNAs from mixed aged rat brains enhanced the chances of identifying related clones since from the Northern blot it was known that clone OL0755 related mRNAs were expressed at P1 and P10. Two clones, OL0755-A and OL0755-B, were isolated from this library.

Sequence analysis of clones OL0755-A and OL0755-B showed them to be closely related. The pattern of similarity between them suggests that they are likely to have arisen from the alternative splicing of a primary transcript encoded by a common gene. Alternative splicing is a common phenomenon amongst proteins involved in myelination. MBP has at least six different isoforms which are all products of alternative splicing mechanisms. PLP, MAG and CNPase also use alternative splicing as a way to increase protein variation in the nervous system. Related proteins with the same N-terminus and different C-termini, as occurs with clones OL0755-A and OL0755-B, is also not unusual as illustrated by MOBP.

The relationship of clones OL0755-A and OL0755-B with clone OL0755 is less straightforward. Comparing clone OL0755 with OL0755-B, it appears that three sections of sequence present in OL0755-B are missing from OL0755; a large section of about 600 bp towards the 3′ end, a very small 10 bp section towards the 5′ end and the 5′ terminal sequence. These identical sections of sequence are, however, present in clone OL0755-A, although they are also interspersed by sequence unique and specific to clone OL0755-A. There are two possible explanations for the relationship between OL0755 and the other two clones. The first is that OL0755 represents the partial 3′ sequence of a third related clone. This clone could possibly correspond to a signal at around 4 kb on the Northern blot probed by OL0755, although it has been speculated that this signal is due to non-specific binding by the ribosomal subunit. If clone OL0755 does represent another 4 kb variant then a further 3 kb of 5′ sequence is missing. It would also be much more difficult to explain the relationship of all three clones by alternative splicing mechanisms. The second possibility, which is probably the more plausible, is that a mistake occurred during the construction of the original cDNA libraries used for the subtractive hybridisation. During the reverse transcription of isolated polyA RNA sequences the reverse transcriptase enzyme may have skipped over some of the sequence resulting in a truncated cDNA clone with sections missing. If this has occurred then clone OL0755 is probably an artificially truncated form of clone OL0755-B.

4.4 Evidence that clones OL0755-A and OL0755-B correspond to the mRNAs identified on the Northern blot and that they are full length cDNAs.

The sizes of clones OL0755-A and OL0755-B approximate to the sizes of the mRNAs identified on the Northern blot probed by OL0755. Hence they are likely to represent full length cDNAs. Clone OL0755-A, from sequence analysis is 2771 bp in length and clone OL0755-B is 2050 bp. These correlate with signals at

approximately 3 kb and 2 kb respectively on the Northern blot. An exact estimation of the sizes of the mRNAs identified on the Northern blot is difficult since more accurate size markers were not available. Additional evidence that these two clones correspond to the signals on the Northern blot is that probe OL0755-A(S), derived from the 600 bp fragment of sequence specific to clone OL0755-A, clearly identified a single mRNA molecule corresponding to the band at approximately 3 kb. Since there is only a very small fragment of sequence which is specific to clone OL0755-B (approximately 50 bp) is was not possible to repeat this experiment to show that clone OL0755-B directly correlates with the smaller signal at about 2 kb on the Northern blot. However, evidence is also provided by the anti-peptide antibody Ab755 which identifies two proteins with sizes corresponding to those predicted from sequence analysis of clones OL0755-A and OL0755-B. It was predicted that clone OL0755-A encoded a protein of about 60 kD (59690 daltons) and clone OL0755-B encoded one of about 50 kD (49252 daltons). On a Western blot two proteins with comparable sizes are identified by Ab755. Therefore the evidence supports the contention that clones OL0755-A and OL0755-B are full length cDNA clones encoding the mRNAs identified on the original Northern blot.

4.5 What is known about the proteins encoded by clones OL0755-A and OL0755-B?

When the sequences were compared with the Genbank and EMBL databases, the only significant similarity was a 95.6% identity of 201 nucleotides (bp 1956-2157 in clone OL0755-A, which is also present in clone OL0755-B, bp 1247-1448) with a R. norvegicus brain 262 bp cDNA fragment named clone sap37f which was isolated from an expression cDNA library with antisera raised against a synaptic protein preparation, the postsynaptic density fraction (Langnaese et al., 1996). It is possible therefore that the proteins encoded by clones OL0755-A and OL0755-B could be associated with synaptic elements of the CNS.

The predicted sequences of the two proteins provide little information about possible function. From the hydropathy profiles performed on the translated proteins as predicted by sequence analysis of the cDNA clones, both proteins are mostly hydrophilic. The 60 kD protein encoded by clone OL0755-A has a 13 amino acid hydrophobic segment with predicted beta-sheet secondary structure. In theory it is possible for an extended beta strand of only nine residues to span a lipid bilayer (Gardinier et al., 1992), so this protein could be a transmembrane protein. The other 50 kD protein has a region of 20 amino acids with potential α-helix structure which could also span a plasma membrane. However the potential glycosylation sites,

identified by analysis of the predicted proteins, appear not to be utilised as demonstrated by the deglycosylation experiments and this suggests that neither of the proteins are integral membrane proteins. It is not known whether the proteins are phosphorylated since there was no shift in mobility of the proteins on an electrophoresis gel following dephosphorylation. A shift in mobility following dephosphorylation confirms that a protein is phosphorylated but when there is no shift in mobility the possibility cannot be excluded.

4.6 Expression patterns

mRNA expression for both clones is developmentally regulated as indicated by a developmental Northern blot. Both clones show peak expression at P21, although the smaller clone, OL0755-B, is consistently more abundant than the larger clone, OL0755-A, throughout development. When protein expression is analysed on a developmental Western blot, the abundance of the smaller protein encoded by clone OL0755-B increases during development peaking at P21 in complete agreement with the developmental Northern. However the abundance of the larger protein encoded by clone OL0755-A appears to decrease as development progresses. The developmental profiles of both mRNA and protein expression correlate with a potential role in the development of the nervous system. Neurogenesis primarily occurs prior to birth in most regions of the brain. Gliogenesis of oligodendrocytes is principally a postnatal event while for astrocytes it occurs during late foetal development and postnatally. Since expression of clones OL0755-A and OL0755-B is detected at P1 and they remain constitutively expressed in adults, the encoded proteins may be important in interactions between glial cells and neurons, perhaps also in the process of myelination.

When ISH studies were performed on P21 rat sagittal brain sections, an age corresponding to maximum expression of the mRNAs for clones OL0755-A and OL0755-B, using clone OL0755 as the probe, since its sequence is common to both OL0755-A and OL0755-B, strong expression in the granule cell layer of the cerebellum, the cerebellar nuclei and in the hippocampus indicated a predominantly neuronal pattern of expression. The granule cell layer of the cerebellum contains numerous small neurons with non-myelinated axons which pass outwards to the molecular layer and synapse with the dendrites of Purkinje cells. It is in the granule cell layer that mRNA expression is strongest.

On closer inspection, however, a few cells in the molecular layer of the cerebellum also appear to express mRNA detected by clone OL0755. This would seem to indicate expression by glial cells, although at a lower level than by neurons.

Further evidence for glial cell expression is present in the forebrain when the brain sections were double labelled immunocytochemically with antibodies identifying oligodendrocytes and astrocytes. Although morphological preservation of these cells in the section is far from ideal, there do appear to be some cells positively labelled with an anti-MBP antibody (oligodendrocytes) which are also labelled by ISH, and other cells labelled by an anti-GFAP antibody (astrocytes) which are also indicated by ISH labelling. The expression of mRNA, identified by clone OL0755, in glial cells is further supported by the ISH studies on rat optic nerve. Compared to the sense control, clone OL0755 is definitely identifying mRNA present in optic nerve. Since, as previously discussed, it is widely accepted that the optic nerve does not contain neuronal cell bodies then these signals must arise from expression by glial cells. This is in agreement with the results from ISH studies on cultured glial cells and Northern blotting of optic nerve. Therefore it appears that mRNAs identified by clone OL0755 are expressed predominantly by neurons but also to a lesser extent by glial cells.

When the mRNA expression pattern specific to clone OL0755-A was investigated using OL0755-A(S) as a probe, a very similar pattern was observed to that when OL0755 was the probe. Expression was predominantly neuronal again with strongest expression in the granule cell layer of the cerebellum. Signal was quite intense in the hippocampus and there was also some labelling of cerebellar nuclei. Considering that clone OL0755-A mRNA is less abundantly expressed than OL0755-B as deduced from Northern blots, a weaker signal is expected. When the same investigation was done on optic nerve, a signal, significantly weaker than that detected by OL0755 was produced. It therefore appears that the expression pattern for clone OL0755-A, although less intense, is the same as that detected by clone OL0755. Unfortunately, as previously discussed, it is not possible to repeat this experiment with a probe specific to clone OL0755-B so it is unknown whether the expression pattern for this clone is different. It could be that clone OL0755-B mRNA is expressed only by one specific cell type as opposed to expression by neurons and glia as demonstrated by OL0755-A. However when the optic nerve is probed by OL0755, which is common to both OL0755-A and OL0755-B, the signal is significantly stronger than when the probe is specific to clone OL0755-A (probe OL0755-A(S). This indicates that mRNAs in addition to those encoded by clone OL0755-A are expressed by glial cells in optic nerve and probably correspond to mRNAs encoded by clone OL0755-B. Therefore it seems likely that mRNA encoded by clone OL0755-B is expressed at least by glial cells.

Although ISH studies indicate strong expression in neurons and weaker

expression in glial cells at P21 by clones OL0755-A and OL0755-B this balance of expression may be different at different ages of development. It could be that at earlier ages and at times corresponding to active myelination there is a shift of expression towards glial cells. Further ISH analyses on brain sections of different ages are required to investigate this possibility.

Attempts were made to investigate the expression patterns of the proteins in sagittal brain sections using Ab755 but they proved to be unsuccessful possibly because the epitope is masked somehow by the conformation of the native proteins. Unsuccessful attempts were also made to denature the proteins in situ and expose the reacting epitope. Therefore it is not possible at this stage to identify the cellular locations of these proteins. Work is in progress to generate a fusion protein which can be used as an antigen. The protein is more likely to be in its natural conformation and therefore enable an antibody to be raised against an accessible epitope. Studies can then be carried out to define the cellular localisations of the proteins which may assist in the elucidation of function.

4.7 Proposed future work

As already discussed, future work should investigate mRNA expression of each clone in brain sections at different ages to deduce whether there is a shift in expression towards glial cells at an earlier age. Once a suitable antibody has been raised, investigations exploring the expression patterns of the encoded proteins can then proceed. Possibly an antibody specific for the protein encoded by clone OL0755-A can also be produced. Localisation studies and in situ developmental profiles of the proteins may suggest potential roles.

Future work could also include the identification of the gene encoding clones OL0755-A and OL0755-B. Once the encoding gene has been identified, gene disruption experiments could be performed in a bid to gain information about the function of the encoded proteins. Fractionation experiments could be performed to investigate interactions of the proteins with other components of the nervous system. Identifying binding partners of the proteins may also suggest potential function. The similarity of a small part of the clones with sap37f which was isolated from an expression cDNA library with antisera raised against a synaptic protein preparation (Langnaese et al., 1996) may indicate association of the encoded proteins with synaptic elements, perhaps as part of some signalling system or transport mechanism. Investigating potential interactions of the proteins with synaptic elements may therefore be important. Alternatively interactions with cytoskeletal elements or extracellular matrix molecules could implicate a structural role. It is

also possible that these proteins have enzymatic activity and may contribute to the metabolism of cells in the nervous system. Further investigations in the future will eventually provide answers about these potentially important and interesting proteins expressed in the developing CNS.

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